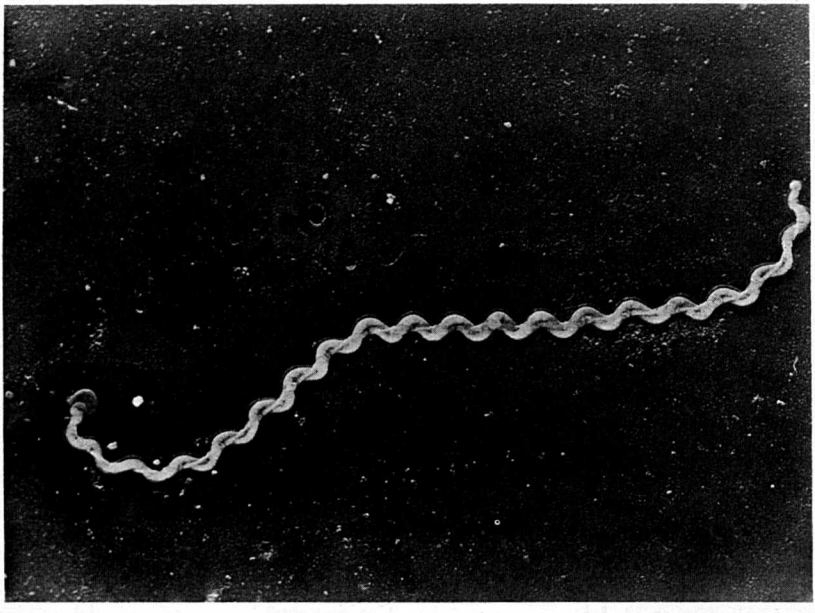

*Leptospira icterohaemorrhagiae.* Spray preparation shadowed with chromium. Magnified 12,500×.

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# LEPTOSPIROSIS

## Epidemiology, Clinical Manifestations in Man and Animals, and Methods in Laboratory Diagnosis

*by*

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# PREFACE

In an editorial nearly 8 years ago, Beeson predicted that human leptospirosis would be recognized more frequently in America and that further knowledge of the varied clinical forms would be acquired. The validity of this prediction is reflected in our current knowledge. In fact, a rather marked increase appears to have occurred in the known prevalence of leptospirosis in both the human and domestic animal population in the United States during this period as shown in the figures in this manual. This increase may be accounted for by a combination of at least three factors. Perhaps one of the most important has been the development and application of simpler techniques in the laboratory confirmation of disease and the availability of these diagnostic services in laboratories throughout the country. Contrary to common beliefs, leptospires can be cultivated readily by simple procedures that are quite practical for hospital and public health laboratories. Similarly, the development of reliable rapid serologic tests have simplified diagnostic procedures and made possible extensive epidemiological strides.

Possibly the second outstanding factor has been a greater awareness of the disease and its many variable clinical factors. Recent information indicates that leptospires are involved in many cases of aseptic meningitis and are not an infrequent cause of "fever of unknown origin" (FUO). An increasing number of laboratories are now testing serums for leptospiral antibodies which were submitted for viral serology and found negative. This practice has been highly productive.

Of equal importance is the epidemiological picture. It has been established that the epidemiology of the leptospiroses will follow a characteristic pattern based primarily upon the fact that they are zoonoses, diseases transmitted from animal to animal and from animal to human. Although this basic pattern has not changed, the apparent existence of an epizootic or panzootic period of the leptospiroses suggests changing environmental conditions which are more favorable to the survival of leptospires.

Many new wild animal hosts of leptospires are being found and, at present, our knowledge of the nature of the infection, the duration of the carrier state, and their role in the epidemiology of leptospirosis is limited. We must assume that they play an important part in the chain of transmission until proven otherwise. Obviously, further research is needed to clarify the situation.

To contain these infections and prevent their spread to man, it is important that health workers know how to recognize and identify the

disease when it appears. This booklet, "Leptospirosis—Epidemiology, Clinical Manifestations, and Methods of Laboratory Diagnosis," is an excellent summation of the present practical knowledge available. The authors, Mildred M. Galton, Robert W. Menges, Emmett B. Shotts, Jr., A. J. Nahmias, and Clark W. Heath, have had considerable experience with leptospirosis, both in the laboratory and field.

Our present knowledge of the epidemiology of leptospirosis is such that there is little likelihood that the disease can be eradicated or controlled in the wild animal hosts which may serve as sources of infection for man and other animals. It is a disease that will be a problem for both public health and animal health authorities for years to come.

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## Section I

### DISTRIBUTION IN THE UNITED STATES

Little progress was made in the study of leptospirosis in the United States for some 35 years after Stimson (*1*) first observed leptospires in sections of kidney from a patient who had died in 1905 of an illness believed to be yellow fever. *Leptospira icterohaemorrhagiae* was isolated from rats in New York City by Noguchi in 1917 (*2*), and 5 years later, the first human infection with *L. icterohaemorrhagiae* was reported by Wadsworth (*3*). A review of the American literature by Larson in 1941 (*4*) revealed 93 reported cases of Weil's disease. Infections with *L. canicola* were recognized in dogs in 1937 and man in 1938 by Meyer *et al.* (*5*, *6*) in California. In recent years, many sporadic human cases of "canicola fever" and two rather large waterborne epidemics (*7*, *8*) have occurred. Infections in dogs are widespread as evidenced by the estimate that 25 percent of dogs in this country now have leptospirosis or have already had it, and 25 to 50 percent of these animals become temporary carriers (*9*).

Leptospires in tissues of cattle were observed in 1944 by Jungherr (*10*) in Connecticut. Four years later, they were first isolated from bovine cases of leptospirosis in New Jersey by Baker and Little (*11*) and identified as *L. pomona* by Gochenour *et al.* (*12*). About this time, Beeson (*13*) reported the first case of "aseptic meningitis" due to *L. pomona* infection. The following year, a waterborne outbreak attributed to *L. pomona* and involving about 50 persons was reported by Schaeffer (*14*) in Alabama. In addition, three outbreaks occurred in Georgia between 1940 and 1949 (*15*, *16*) that were diagnosed in retrospect. More recently, two small outbreaks in which *L. pomona* was implicated occurred in Florida (*17*) and South Dakota (*18*) and a large outbreak involving more than 40 persons was investigated in Iowa (*19*). In this most recent outbreak in Iowa, *L. pomona* was isolated from both human cases and from the urine of cattle that had access to the stream where the patients had been swimming. All of these waterborne leptospiral outbreaks followed a similar pattern. They occurred in the middle or late summer; there was presumed contamination of a stagnant pond or slow-moving creek by urine from infected animals, and subsequent transmission to humans resulted from immersion in the contaminated water. The patients were young, primarily children ranging in age from 5 to 16 years, but with some young adults.



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An outbreak of pretibial fever occurred among troops at Fort Bragg in August 1942, and again in the summers of 1943 and 1944 (*20, 21, 22, 23*). All of the individuals involved had been quartered in the same general area of the post. Many had gone swimming in nearby ponds, but, since not all had done so, the source of infection remains obscure. The reported outbreaks in the United States are summarized in table 1.

In recent years, reported human cases of leptospirosis in the United States have increased. Cases reported from 1905 to 1948 were summarized by Molner *et al.* (*24*) according to geographic distribution. During this 43-year period there were 306 cases in 25 States, and 78 (25 percent) of these cases were from Michigan. In contrast, a summary of cases on which the Communicable Disease Center or the Division of Veterinary Medicine, Walter Reed Army Institute of Research, received information during a 7-year period (1953–60) revealed a total of 601 cases in 45 continental States. In addition, 147 cases occurred in outbreaks reported between 1951 and 1960. The distribution of these cases is shown in figure 1 (*25*).

The primary problems in the United States appear to be with the more common types, *L. icterohaemorrhagiae, L. canicola,* and *L. pomona,* in domestic animals and humans. However, nine other serotypes have been isolated and serologic findings suggest the presence of several other types. *L. autumnalis,* the cause of "Fort Bragg fever," was not identified as such until 8 years after the initial outbreak (*20*). Six years later, the first animal host for this type in the United States was detected when the organisms were isolated from the kidneys of raccoons trapped in southwest Georgia (*26*). *L. ballum* has been found frequently in rural house mice and opossums and occasionally in rats and laboratory mice (*27, 28*). Clinical and serologic evidence of human infection due to *L. ballum* contracted from Swiss white mice has occurred among seven laboratory workers at the Rocky Mountain Laboratory, Hamilton, Mont. (*29*). The wide range of hosts that may harbor leptospires became even more apparent with the recent isolation of *L. ballum* from an eastern hog-nosed snake (*Heterodon platyrhinus*) caught in a mousetrap in a cattle shed in Illinois (*30*).

During cultural studies (1954–58) at the Communicable Disease Center, Newton, Ga., Field Station on wild mammals, *L. grippotyphosa* was isolated from raccoons trapped within 60 miles of a Florida farm on which cattle showed antibodies to *L. grippotyphosa* in 1952 (*31, 32*). In addition, these studies have revealed the presence of *L. pomona, L. australis, L. autumnalis,* two serotypes that belong to the hyos serogroup, one of which has been designated as *L. hyos bakeri* (*33*), the other as *L. atlantae* (*34*), and a new subserotype of the hebdomadis serogroup, *L. mini georgia* (*35*) in wild mammals. One laboratory acquired human infection (*36*) with *L. mini georgia* has occurred and serologic evidence has suggested another. It is notable that 61 percent of the leptospiral

TABLE 1

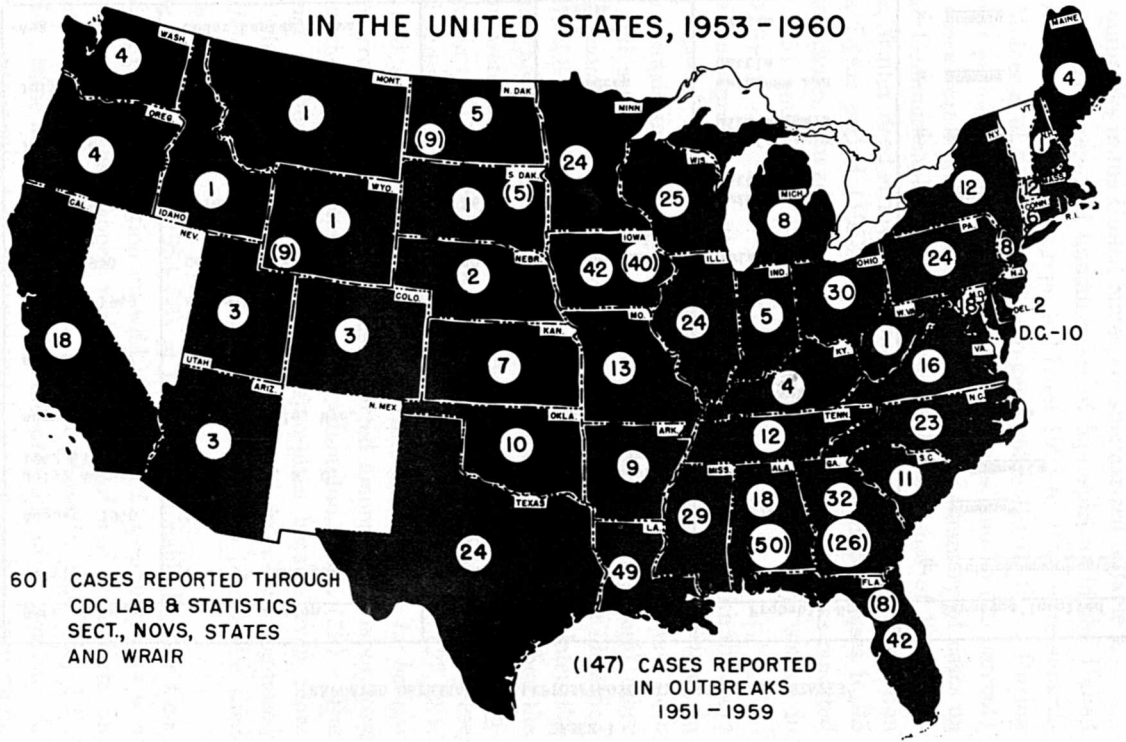
REPORTED OUTBREAKS OF LEPTOSPIROSIS IN THE UNITED STATES[1]

| Date of Onset | Location | No. of Cases | Age Range | Probable Source | Serotype Involved |
|---|---|---|---|---|---|
| July, 1939 | Near Philadelphia, Pa. | 7 | Young adult males | rats ? | L. icterohaemorrhagiae[2] |
| August, 1940 | Wrens, Ga. | 35 | Av. 16 | cattle | L. pomona* |
| July, August, 1942-43-44 | Ft. Bragg, N. C. | 40/yr. | Young adults (soldiers) | unknown | L. autumnalis |
| August, 1942 | Jackson Hole, Wyo. | 24** | 15-19 | dogs, deer or other animals | L. canicola* |
| August, 1947 | Calvary, Ga. | 10 | 13-24 | dead mule in creek ? | ? |
| August, 1949 | Swainsboro, Ga. | 12 | Young adults | cattle | L. pomona* |
| July, 1950 | Geneva, Ala. | 50 | Adolescents & young adults | swine | L. pomona* |
| July, 1952 | Columbus, Ga. | 26 | 5-20<br>2 adults | swine, dogs, cattle | L. canicola |
| July-Aug. 1956 | Wakpala, S. D. | 3 | Young adults | cattle, swine, or wild animals | L. pomona* |
| July, 1958 | Madison, Fla. | 8 | Children | swine*** and cattle | L. pomona |
| Aug., 1959 | Cedar Rapids, Iowa | 42 | Children & adults | cattle | L. pomona |

1. With the possible exception of the Fort Bragg outbreaks, all were attributed to swimming in contaminated water.
   *Serological evidence only.
   **9 confirmed serologically
   ***L. pomona isolated from swine, serologic evidence in cattle and human cases.
2. Organisms recovered from blood of 1 fatal case.

Figure 1

## OCCURRENCE OF HUMAN LEPTOSPIROSIS IN THE UNITED STATES, 1953 - 1960




601 CASES REPORTED THROUGH CDC LAB & STATISTICS SECT., NOVS, STATES AND WRAIR

(147) CASES REPORTED IN OUTBREAKS 1951 - 1959

isolates from skunks proved to be *L. pomona.* Of further interest was the isolation of *L. pomona* from 2 skunks in a group of 18 animals trapped on a Georgia farm where 67 percent of a herd of 96 beef cattle had high agglutinin titers to *L. pomona* and 5 of the cows had aborted.

The possibility of an animal host of *L. autumnalis* was not investigated during the Fort Bragg outbreaks or after the causative agent was established. However, in October 1957, a limited study (*37*) yielded leptospires from the kidneys of 10 (11.7 percent) of 84 wild animals trapped in this area. The infected animals included five raccoons, three gray foxes, and two red foxes. Three of the leptospiral strains isolated belong to the australis serogroup and seven are *L. grippotyphosa.* While an animal host of *L. autumnalis* was not established during this limited study, leptospiral infection among the wild animal population appeared more prevalent than in the southwest Georgia area. Serologic evidence of human infection with a member of the australis serogroup has been reported (*38*).

In Louisiana, Roth *et al.* (*39*) has found leptospires in 41 percent of 227 wild animals. More than 50 percent of the skunks were harboring leptospires. Although identification of all types isolated had not been completed, the preliminary report included *L. pomona, L. canicola, L. ballum,* and members of the icterohaemorrhagiae, hyos, and hebdomadis serogroups. In addition, serologic evidence suggests the presence of *L. bataviae* infection in humans (*40*), *L. sejroe* in cattle (*32, 41*), and *L. grippotyphosa* in humans (*42*) and cattle (*32*). Although *L. sejroe* has not been isolated from cattle in the United States, several strains of a closely related serotype, *L. hardjo,* have been obtained in Louisiana (*43*), Nebraska (*44*), and Pennsylvania (*45*), from animals both with and without clinical signs of disease.

## EPIDEMIOLOGY

The epidemiology of the leptospiroses has been shown to follow a characteristic pattern based, in part, upon the fact that they are diseases transmitted from animal to animal and from animal to humans. The chain of transmission, with rare exception, stops with human infections, although Gordon Smith and Turner (*46*) suggest that in Malaya man may be important in introducing infection to previously uninfected places and host communities.

For many years, rats and dogs were considered to be the primary animal carriers, but, as the search for leptospires continues, the host range broadens. While leptospirosis is still prevalent in dogs and infection in rats ranges from 30 to 60 percent, the disease is now a major problem in cattle and swine, and, in some areas, sheep, goats, and horses become infected. Further problems are presented by increasing evidence that indicates a wide distribution of leptospires in a variety of wild animals throughout the world.

The rat is one of many rodent carriers including mice and voles (*47, 48, 49*). Other wild animal reservoirs include bats, mongooses, shrews, bandicoots (*50*), jackals (*51*), and hedgehogs (*52*). In addition, in the United States, opossums, raccoons, skunks, foxes, wildcats, beaver, nutria, armadillos, and rabbits have been infected (*31, 39*). In these host animals, leptospires will localize in the kidneys and may be found in the lumina of the convoluted tubules. After acute or even inapparent infection, these animals may become carriers and shed the organisms in their urine for long periods. The infected urine of these shedders serves as an important source of infection in man and other animals.

Certain leptospiral serotypes appear to have a primary animal host, but they may infect other animals. It is not unusual for a so-called primary host to become infected with other serotypes (*53*). For example, *L. canicola,* found principally in dogs, has been isolated from cattle (*54, 55*), swine (*56*), jackals (*51*), hedgehogs (*52*), and skunks (*57*), and serologic evidence suggests it may infect raccoons (*58*), while dogs have been found to harbor at least nine other serotypes, including *L. pomona* (*59, 60, 61*). Reported isolations of different leptospiral serotypes in the United States and their hosts are presented in table 2 (*25*).

## TRANSMISSION TO MAN

In the transmission of leptospiral infections to humans, these animal carriers, which may become urinary shedders after acute, mild or, more frequently, inapparent infection, serve as important foci. A diagram illustrating the current pattern of the transmission of leptospirosis in the United States is presented in figure 2. Infection of humans and other animals results from direct or indirect contact with infected urine of these shedders. For example, direct contact may occur when individuals care for sick animals, fondle a pet dog, or handle the tissues of infected animals in abattoirs. Indirect contact occurs when the organisms are excreted in water or moist soil and individuals are subsequently exposed while swimming, working, or otherwise coming in contact with the contaminated environment.

The role of arthropod vectors in the transmission of leptospirosis has been studied both experimentally and in nature. Several investigators (*62, 63*) have been able to infect ticks by allowing them to feed on infected guinea pigs or hamsters. These experimentally infected ticks transmitted the disease to normal animals. The isolation of *L. grippotyphosa* from the European tick, *Dermacentor marginatus* S., has been reported from Russia (*64*). The ticks were from cattle where leptospirosis had occurred among the herd. More recently, Van der Hoeden (*65*) has isolated *L. canicola* from *Rhipicephalus sanguineus* collected from a hedgehog. However, under natural circumstances, he attaches no great importance to the transmission of leptospirosis by ticks in Israel. Nevertheless, ticks should be considered as possible vectors until their significance is known in other areas.

TABLE 2

LEPTOSPIRAL SEROTYPES IN THE CONTINENTAL UNITED STATES

| Serotype | Original Isolation | Known Host | Occurrence | | | |
|---|---|---|---|---|---|---|
| | | | Man | Dogs | Cattle | Swine |
| 1. L. icterohaemorrhagiae | Japan Germany | rat, mouse | common | occasional | ? | ? |
| 2. L. canicola | Netherlands | dog, cattle, swine, skunk | occasional | common | rare | rare |
| 3. L. ballum | Denmark | mice, gray fox, opossum, wildcat, rats, raccoon, striped skunk, spotted skunk, rabbit | rare* | ? | ? | ? |
| 4. L. grippotyphosa | Russia | raccoon, skunk, fox | rare* | ? | rare* | ? |
| 5. L. bataviae*[1] | Indonesia | ? | rare* | ? | ? | ? |
| 6. L. autumnalis | Japan | opossum, raccoon | rare | ? | ? | ? |
| 7. L. pomona | Australia | cattle, swine, skunk, raccoon, wildcat, opossum, woodchuck | common | rare | common | common |

*Serological evidence only

1. L. paidjan, a member of the bataviae serogroup, has recently been isolated from nutria in Louisiana. (Personal communication, Dr. E. E. Roth, Louisiana State University, Baton Rouge, La., 1962).

TABLE 2--(Cont'd.)

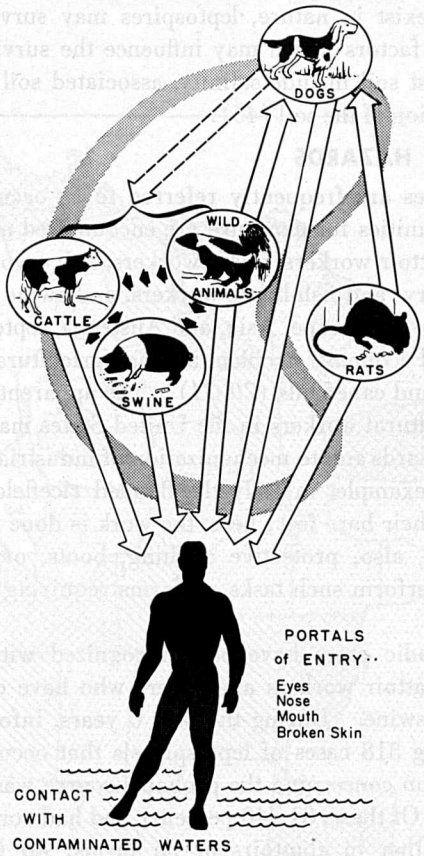
LEPTOSPIRAL SEROTYPES IN THE CONTINENTAL UNITED STATES

| Serotype | Original Isolation | Known Host | Occurrence | | | |
|---|---|---|---|---|---|---|
| | | | Man | Dogs | Cattle | Swine |
| 8. L. sejroe* | Denmark | ? | rare* | ? | sporadic* | ? |
| 9. L. hardjo | Indonesia | cattle | rare* | ? | sporadic | ? |
| 10. L. mini, georgia | United States | opossum, raccoon, skunk | rare | ? | ? | ? |
| 11. L. australis | Australia | raccoon, opossum, fox | rare* | ? | ? | ? |
| 12. L. hyos, mitis J.* | Australia | ? | ? | ? | rare* | ? |
| 13. L. hyos, bakeri | United States | opossum | ? | ? | ? | ? |
| 14. L. atlantae, LT81 | United States | opossum, raccoon, skunk, fox | ? | ? | ? | ? |

*Serological evidence only

Figure 2

## TRANSMISSION of LEPTOSPIROSIS in the U.S.




During the acute phase of leptospirosis in lactating animals, leptospires may be shed in the milk, but no human cases have been attributed to drinking infected milk. This may be explained by Kirschner's (*66*) observation that whole milk is leptospirocidal and the organisms will survive in it only a few hours. However, as pointed out by Mitchell and Boulanger (*67*), the opportunity for spread of the disease from cow to cow by contaminated milking equipment should not be overlooked. In addition, dairy workers should be informed of the hazard in handling and feeding milk from affected cows.

The leptospires usually enter the body through the mucous membranes of the conjunctivae, the nose or the mouth, and through skin abrasions. It is believed, also, that entrance may be through unbroken skin if it is softened by long exposure to water.



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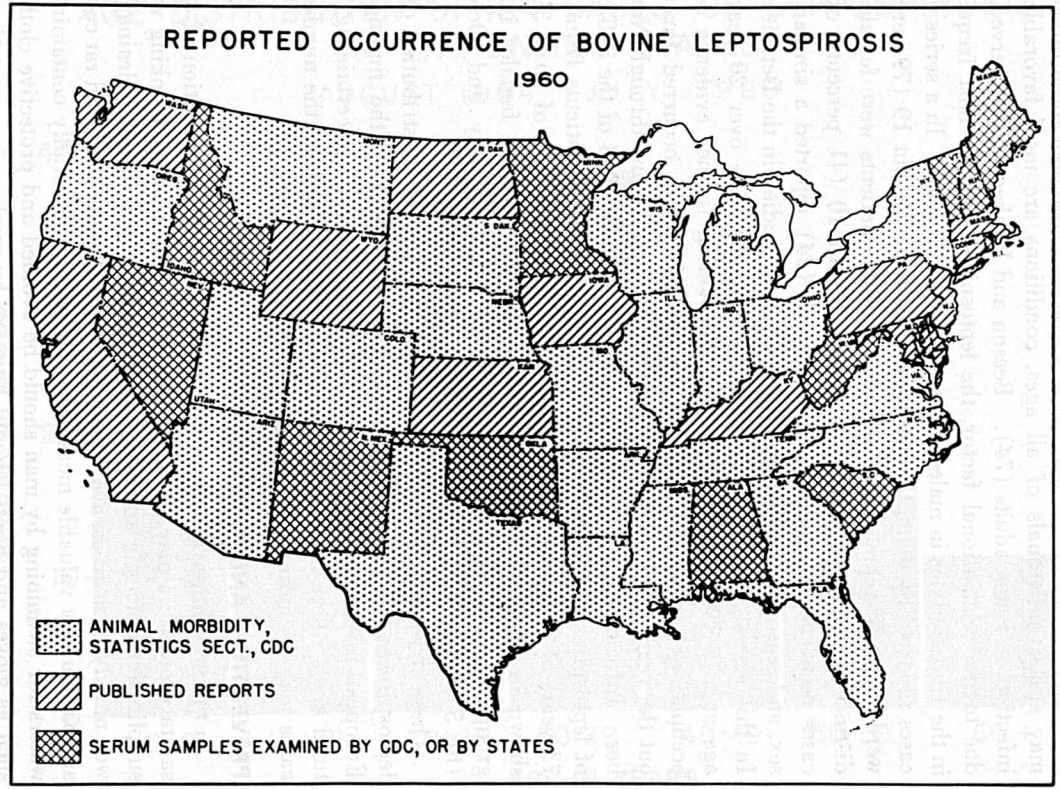
Environments that favor the survival of leptospires outside the body include moist soil, stagnant ponds, or slow-moving streams that are neutral or slightly alkaline; temperatures must be 22° C. or above. When these conditions exist in nature, leptospires may survive several weeks (*68, 69*). Other factors which may influence the survival of leptospires in water and moist soil include salinity, associated soil bacteria, and the chemical constitution of the soil (*46*).

## OCCUPATIONAL HAZARDS

The leptospiroses are frequently referred to as occupational diseases. Certainly, opportunities for exposure are encountered more frequently by veterinarians, abattoir workers, sewer workers, dairy workers, animal husbandrymen, poultry- and fish-house workers, and swine herdsmen here in the United States. In Europe, Asia, and Australia, leptospirosis is an important health and economic problem among agricultural workers, particularly in the rice and canefields (*70, 71*). The apparent lack of this problem among agricultural workers in the United States may be attributed to higher living standards and to mechanization of industrial and agricultural operations. For example, in Italy the flooded ricefields are worked by hand laborers in their bare feet; here, the work is done mechanically. In the United States, also, protective clothing, boots, or heavy shoes are usually worn to perform such tasks on farms requiring contact with contaminated soil.

However, sporadic cases have been recognized with increasing frequency among abattoir workers and others who have close contact with infected cattle or swine. During the past 6 years, information has been obtained regarding 318 cases of leptospirosis that occurred in 37 States (*25*). Information concerning the probable source was obtained on 205 of the 318 cases. Of these, 81 (40 percent) had had contact with infected cattle or swine either in abattoirs or on farms; 40 (19 percent) had been drinking, swimming, or had been accidentally immersed in presumably contaminated water; 32 (15 percent) had had contact with dogs in their homes or in veterinary hospitals; 34 (16 percent) were exposed to rats, 8 (4 percent) to wild animals, and 10 (5 percent) to other animals or possibly contaminated environments in their occupations. The fact that the probable source of more than one-third of these cases was found to be contact with infected cattle or swine may be attributed, in part, to the apparent rapid spread and increase in recognition of bovine leptospirosis in the United States. This disease was reported by York (*72*) to have occurred in only 12 States prior to 1951, whereas, by 1960, it had been recognized throughout the continental States according to data obtained from a summary of animal morbidity reports to the Communicable Disease Center, published reports and serum samples examined by Communicable Disease Center laboratories, as shown in figure 3. In 1954, the U.S. Department of Agriculture, Agricultural Research Service

Figure 3


REPORTED OCCURRENCE OF BOVINE LEPTOSPIROSIS
1960
ANIMAL MORBIDITY, STATISTICS SECT., CDC
PUBLISHED REPORTS
SERUM SAMPLES EXAMINED BY CDC, OR BY STATES

(*73*), estimated that annual losses from bovine leptospirosis were over $112 million, or $25 million greater than losses from bovine brucellosis.

## AGE, SEX, AND SEASONAL PREVALENCE

As a result of these environmental and occupational factors, the epidemiological pattern of human leptospiral infections has certain characteristic features related to age, sex, and season. Although these infections may attack individuals of all ages, conditions are most favorable for infection of young adults (*74*). Beeson and Hankey (*75*) observed that due to the occupational factor, the leptospiroses occur most frequently in the United States in males above the age of 15 years. In a series of 24 cases reported by them, the age range was 10 to 55, but 19 (79 percent) were under 30 years of age and only 5 of the patients were female. A distinct seasonal incidence was observed with 10 (41 percent) of the cases occurring in August. Molner *et al.* (*24*) reported a similar age, sex, and seasonal distribution among 78 cases studied in the Detroit area. In this series, 70 (90 percent) of the patients were over 20 years of age and 73 (93 percent) were male, providing further evidence of an occupational relationship. Although sporadic cases occurred throughout the year, a definite increase was noted from August through November. Information regarding the age distribution of 184 of the 318 cases of leptospirosis previously referred to (*25*) revealed patients from 2 to 73 years of age, but the majority were between the ages of 10 to 39, as shown in figure 4. There were only 32 (12 percent) females in the group. The highest incidence occurred between July and October (fig. 5).

These observations regarding age, sex, and seasonal distribution of the leptospiroses in the United States are in accord with the findings of European investigators, with possibly one exception. In certain agricultural areas where fieldworkers are predominately women, the number of cases may be more numerous in females (*71*).

## PREVENTION AND CONTROL

In the prevention and control of leptospirosis, the application of strict sanitary practices plays an important role. Protection of drinking water supplies for domestic animals from contamination, adequate drainage of wet, muddy farm areas, avoidance of overcrowded feedlots, and rat control are particularly valuable measures. The use of potentially contaminated waters for swimming by man should be avoided and protective clothing, such as gloves and boots, should be worn by workers in occupations of high risk. Thorough cleaning with disinfectant agents, as a sodium hypochlorite solution, effectively eliminates contamination from work areas in poultry- and other food-processing plants.

Leptospiral vaccines were first used in man in Japan in 1933 (*76*). These and subsequent ones tried in Europe caused severe clinical reactions. More recently, formalinized vaccines prepared by a method developed

Figure 4

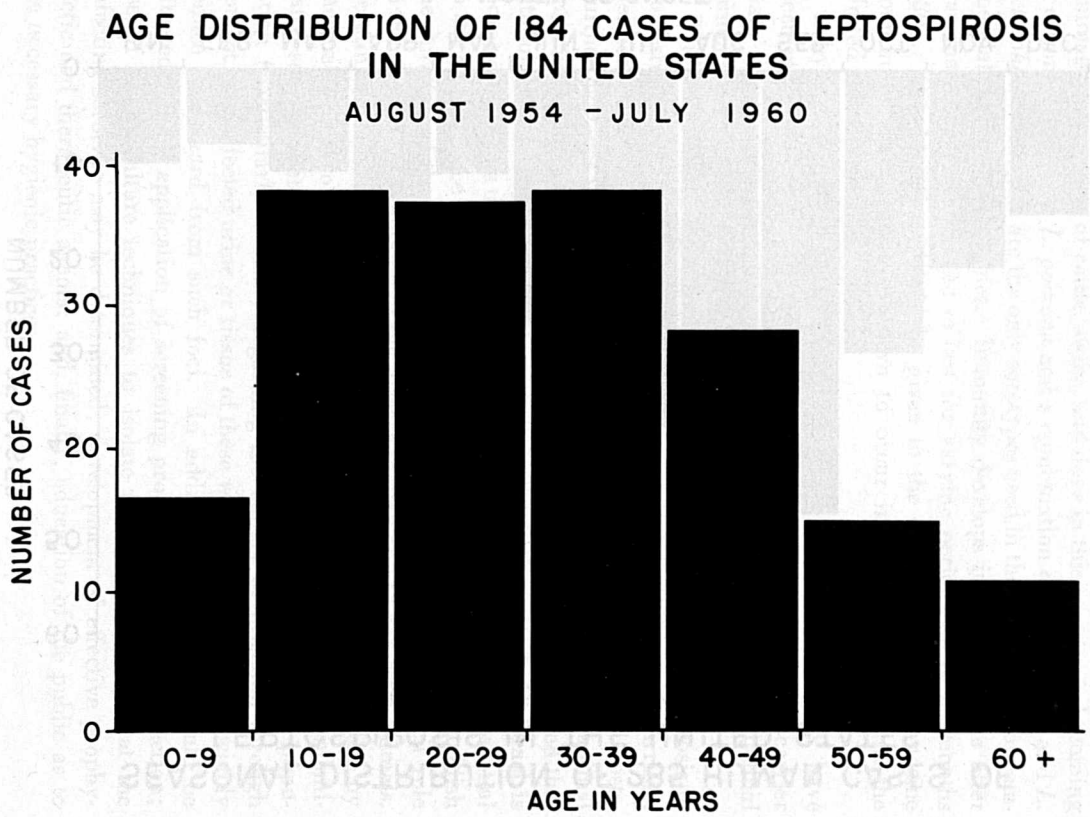
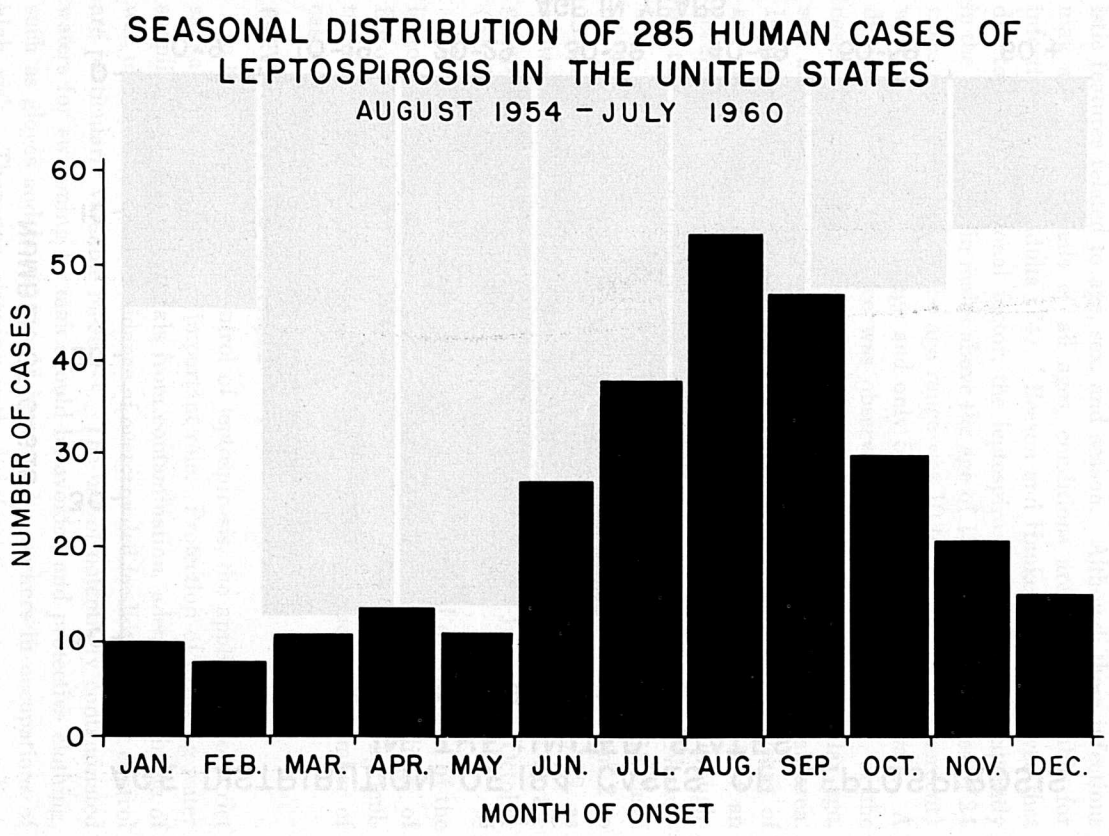

AGE DISTRIBUTION OF 184 CASES OF LEPTOSPIROSIS
IN THE UNITED STATES
AUGUST 1954 - JULY 1960
NUMBER OF CASES
40
30
20
10
0
0-9
10-19
20-29
30-39
40-49
50-59
60 +
AGE IN YEARS

Figure 5


SEASONAL DISTRIBUTION OF 285 HUMAN CASES OF LEPTOSPIROSIS IN THE UNITED STATES
AUGUST 1954 – JULY 1960
NUMBER OF CASES
0
10
20
30
40
50
60
JAN.
FEB.
MAR.
APR.
MAY
JUN.
JUL.
AUG.
SEP.
OCT.
NOV.
DEC.
MONTH OF ONSET

by Babudieri appear to have protected against infection and produced only mild reactions when given to ricefield workers in Italy (*77*) and Spain (*78*) and to agricultural workers in Poland (*79*). Serotypes used to prepare these vaccines were the most prevalent types in the respective area. In the United States, vaccines have not been used in man but with the widespread distribution of leptospirosis in our domestic and wild animals, vaccination of cattle, swine, and dogs in this country is becoming a common practice. *L. pomona* and a combination of *L. canicola*, and *L. icterohaemorrhagiae* are the only serotypes used in the preparation of commercially available vaccines. Immunity develops in 1 to 3 weeks after vaccination and is reported to last for varying periods up to 20 months (*80*). Consideration should be given to the development of a vaccine containing other serotypes known to occur in cattle and swine in the United States.

Dihydrostreptomycin and the tetracyclines (*81, 82, 83, 84, 85*) have been reported by some investigators to successfully eliminate the carrier state but their high cost usually limits their application to individual animals rather than on a herd basis.

While vaccination and the use of antibiotics may control leptospirosis in domestic animals, these methods cannot be applied to wild animal carriers. Neither would it be practical to attempt eradication of wild animal hosts. Further evidence is needed to establish the actual role of these wild animal hosts in the chain of transmission. Van der Hoeden (*86*) has stated that although mutual spread of leptospirosis in domestic animals in Israel is more conspicuous than in wild animals, the latter seem to be more essential to the maintenance of infection. Further study is also needed to establish the duration of the carrier state in these animals and to clarify their role in the possible transmission of the disease to man and to domestic animals. Certainly, establishment of immune domestic animal populations by vaccination, use of disinfectants (*87*) to destroy leptospires in contaminated soil and water in strategic areas and education of the human population (particularly hunters) regarding the danger of infection through contact with infected urine or tissue of these wild animals will appreciably reduce the hazard from such foci. In addition, our efforts should be directed toward application of screening procedures to detect infection; use of direct culture techniques to isolate the leptospires so that the infecting serotype may be determined; development of effective prophylactic and therapeutic agents; and, finally, education of the public as to the necessary hygienic practices.

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## CLINICAL MANIFESTATIONS OF LEPTOSPIROSIS IN MAN

In 1886, Adolf Weil described a severe febrile illness marked by jaundice, hepatosplenomegaly, renal involvement, and nervous symptoms. Since the time of this early description, our knowledge of the clinical manifestations of human leptospirosis has been greatly expanded. The syndrome of Weil's disease can no longer be considered the typical clinical form of leptospirosis. Instead, as medical awareness of the disease has increased and as diagnostic methods have improved, the majority of cases have proven to be anicteric and of less severity than classical Weil's disease.

How frequently human leptospirosis occurs in the United States is not known. However, the disease certainly can no longer be considered "rare" or "exotic." During recent years, an increasing number of sporadic cases (*3–18, 59–61*), as well as several common source outbreaks (*19–24*), of leptospirosis have been reported in the medical literature. In addition, serological studies have indicated that leptospiral infection accounts for a significant proportion (3 to 5 percent) of those illnesses usually diagnosed as "aseptic meningitis" or "fever of unknown origin" (*1, 62, 63*).

The infection of man with leptospiral organisms produces an acute systemic disease, resulting in a wide range of pathological lesions and clinical manifestations (*1–18, 30, 41, 43, 56, 57, 59, 61*). This diversity in symptoms and pathology is reflected in the difficulty with which the disease is diagnosed clinically and in the large number of diseases with which leptospirosis may be confused. Table 3 lists the various organ systems in which leptospiral involvement has been observed. The particular pathological lesions produced are summarized with their associated clinical and laboratory findings, and the most commonly encountered differential diagnoses are noted.

Despite this wide spectrum of clinical pathology, the majority of cases of leptospirosis display a recognizable and self-limited clinical picture. The onset of illness is generally abrupt, following an incubation period which ranges from 2 to 20 days, with an average of 7 days. The ensuing clinical course is often, but by no means always, clearly biphasic (fig. 6). The first phase, lasting somewhat less than a week, is generally marked by chills, high fever, headache, and myalgia. Often conjunctival suf-

fusion is observed in this early stage, and, in more severe cases, hepatic and renal involvement of variable extent are present. This initial phase has been termed "systemic," and it is during these early days of illness that leptospiral organisms are most commonly recovered from the blood and cerebrospinal fluid.

The second phase of illness may vary in length (up to 2 weeks) and generally follows an interlude of several days during which the initial fever and symptoms subside. Fever returns to a milder degree in the second phase and a sterile meningitis is commonplace. Other neurological lesions and uveitis are less common late manifestations. This second phase of illness generally corresponds to the time of increase of leptospiral serum antibodies and has been called the "immune" phase (*57*). Recovery is usually rapid and complete, and both sequelae and relapses of illness seem rare. During the latter phase of illness, patients excrete leptospires in the urine. Occasionally this excretion of organisms continues as a carrier state, but seldom is this observed beyond 2 months after clinical recovery.

The total duration of illness is perhaps related to clinical severity and usually ranges between 1 and 3 weeks. The disease may vary widely in severity, from merely a mild flu-like illness to a fulminating course with jaundice, renal insufficiency, meningitis, and death. The severity of illness seems to depend, in part, on the age and resistance of the patient and, in part, on the "virulence" of the infecting leptospiral strain. Fatalities are rarely seen in the absence of jaundice, and the majority of all cases are anicteric. Most deaths from leptospirosis occur in the presence of advanced hepatorenal involvement.

At least 10 leptospiral serotypes have been associated with human infection in the United States. It has been established that no single serotype produces any single disease syndrome exclusively. Hence, any one of these 10 serotypes can involve one or more of the several organ systems listed in table 3 and can produce disease over a wide range of severity. *L. icterohaemorrhagiae* often causes severe illnesses characterized by jaundice and renal failure, but other serotypes can, on occasion, produce an identical clinical picture of equal severity.

Penicillin, streptomycin, and the tetracyclines have been used by numerous investigators in the therapy of human leptospirosis (*5, 18, 25, 27, 30, 32, 37, 52, 53, 64*), but the value of these antibiotics is still questionable. However, there is suggestive evidence in animals and in humans (*2, 28, 29*) that antibiotic therapy, given in high doses (*34, 36*) within 48 to 72 hours after the onset of illness, is beneficial in reducing the severity of illness. It would therefore appear that speed in suspecting and establishing the diagnosis of leptospirosis is most important if antibiotic therapy is to be of any benefit.

The diagnosis of human leptospirosis requires that the physician be aware of the disease, its clinical manifestations, and its epidemiology.

Table 3

Clinical and Pathological Manifestations of Leptospirosis in Man

| Organs Involved | Pathology** | Clinical Signs and Symptoms | Laboratory Findings | Differential Diagnosis |
|---|---|---|---|---|
| Systemic | Leptospiremia (? Toxemia) | Fever: Often biphasic, may be accompanied by relative bradycardia. Chills, sweats, malaise, prostration, anorexia, nausea and vomiting, joint pains, sore throat, weight loss. | WBC: Normal or elevated, usually with shift to left. ESR: Elevated. Anemia: normochromic and normocytic. Leptospires may be isolated from the blood during first week after onset. Antibody titers rise thereafter. | Influenza<br>Q fever<br>Tuberculosis<br>Rheumatic fever<br>Brucellosis<br>Rat-bite fever<br>Epidemic hemorrhagic fever<br>Malaria<br>Relapsing fever |
| Muscle | Swelling of muscle fibers with loss of cross striations, vacuolation and hyalinization, infiltration with histiocytes, granulocytes, and plasma cells. Leptospires demonstrable by silver stains. | Pain and tenderness, especially of muscles of extremities and back. Muscle weakness and stiffness. Abdominal pain often due to involvement of abdominal muscles. | Muscle biopsy suggestive | Collagen diseases |

*NOTE: This table has been compiled from the literature to demonstrate the numerous pathological lesions, clinical signs, and laboratory findings that may be present with leptospiral infection. In addition, this table lists those diseases which may be confused with leptospirosis. In any single case of leptospirosis, of course, only a few of the many clinical manifestations listed may appear. Those organ systems listed first are, in general, most commonly involved.

**References 2, 30, 39, 40, 44-49, 54, 55, 57, 58.

Table 3--2

| Organs Involved | Pathology | Clinical Signs and Symptoms | Laboratory Findings | Differential Diagnosis |
|---|---|---|---|---|
| Meninges and brain | Edema and inflammatory infiltration of leptomeninges.<br>Small hemorrhages in various areas of the brain occasionally.<br>Perivascular round cell infiltrations rarely. | Headache, stiff neck, meningismus.<br>Transient paralysis, dizziness, convulsions occasionally.<br>Delirium, disorientation, psychoses rarely. | CSF pressure may be elevated.<br>CSF cells (early, granulocytes; late, lymphocytes) rarely over 1000.<br>CSF protein elevated (rarely over 100 mg%)<br>CSF sugar normal.<br>Fluid may be bile stained.<br>Leptospires may be isolated from CSF during first week. | Poliomyelitis<br>Aseptic (viral) meningitides<br>Bacterial meningitides<br>Tuberculosis meningitis<br>Encephalitides (Various etiologies)<br>Toxoplasmosis |
| Kidneys | Interstitial inflammatory infiltration and edema of the cortex and medulla with degenerative tubular changes, especially of the epithelium of convoluted tubules.<br>Leptospires demonstrable by silver stains at all levels of renal tubules. | Dysuria, costovertebral pain and tenderness.<br>Oliguria and anuria.<br>Uremia | Albuminuria.<br>Hematuria and pyuria.<br>Casts (hyaline, granular, cellular, bile)<br>Low specific gravity<br>Elevated BUN, NPN<br>Electrolyte imbalance.<br>Leptospires may be isolated from urine one week after onset. | Acute pyelonephritis<br>Acute glomerulonephritis<br>Acute tubular necrosis |
| Liver | Dissociation of liver cords.<br>Portal infiltration<br>Cloudy swelling of liver cells with frequent double and giant nuclei.<br>Inspissated bile in canaliculi.<br>Kupffer cells enlarged.<br>Scattered foci of hepatic cell necrosis.<br>Leptospires demonstrable by silver stains. | Jaundice<br>Liver tenderness<br>Hepatomegaly<br>Abdominal pain | Liver function tests abnormal. (Pattern consistent with hepatocellular type of jaundice)<br>Bilirubin elevated<br>Cephalin Flocculation elevated<br>Thymol turb. elevated<br>Alkaline phosphatose elevated<br>A/G ratio altered<br>Liver biopsy not diagnostic | Infectious hepatitis<br>Serum hepatitis<br>Yellow fever<br>Acute hepatic necrosis<br>Obstructive jaundice |

Table 3--3

| Organs Involved | Pathology | Clinical Signs and Symptoms | Laboratory Findings | Differential Diagnosis |
|---|---|---|---|---|
| Eyes | Early: Conjunctival suffusion<br>Episcleritis<br>Subconjunctival hemorrhage<br>Late: Uveitis(iridocyclitis, chorioretinitis)<br>Optic neuritis<br>Retinal hemorrhage | Photophobia, burning of eyes, retro-orbital pain, papilledema, diplopia, strabismus, ptosis.<br>Scleral icterus | Leptospires may be isolated by anterior chamber puncture | Early symptoms may be seen with other acute toxic febrile illnesses<br><br>Uveitis (other causes) |
| Skin | Macular or maculopapular dermatitis (May be morbilliform, urticarial) | Pretibial rash (May also occur over any area of body, especially trunk) | | Typhoid fever<br>Allergic rashes<br>Syphilis<br>Rickettsial diseases<br>Measles<br>Scarlet fever<br>Meningococcemia<br>Enteroviruses |
| Lungs | Intra-alveolar and interstitial hemorrhage.<br>Congestion and edema | Cough (may be productive)<br>Hemoptysis<br>Rales with signs of congestion or consolidation. | X-ray: Small, patchy, localized, widely disseminated infiltrations.<br>Larger confluent areas of consolidation | Pneumonitis:<br>bacterial<br>viral<br>mycotic<br>Pulmonary edema |
| Vasculature | Hemorrhages in serous membranes, skeletal muscles, gums, skin, liver, kidneys, lungs, GI tract. | Hemoptysis, hematemesis, melena, epistaxis, petechia, purpura, hematuria, subconjunctival hemorrhage. | Usually normal bleeding, clotting, and prothrombin times.<br>Platelet normal.<br>Stool guaiac positive.<br>Hematuria<br>Normochromic, normocytic anemia | Blood dyscrasia |
| GI tract | Hemorrhages in stomach and small intestine, interstitial pancreatitis | Anorexia, nausea and vomiting, abdominal pain (may be due to abdominal myositis).<br>GI hemorrhage<br>Diarrhea, constipation (latter more common). | Stool guaiac positive.<br>Amylase elevated | Cholecystitis<br>Appendicitis<br>Pancreatitis<br>"Surgical abdomen" |

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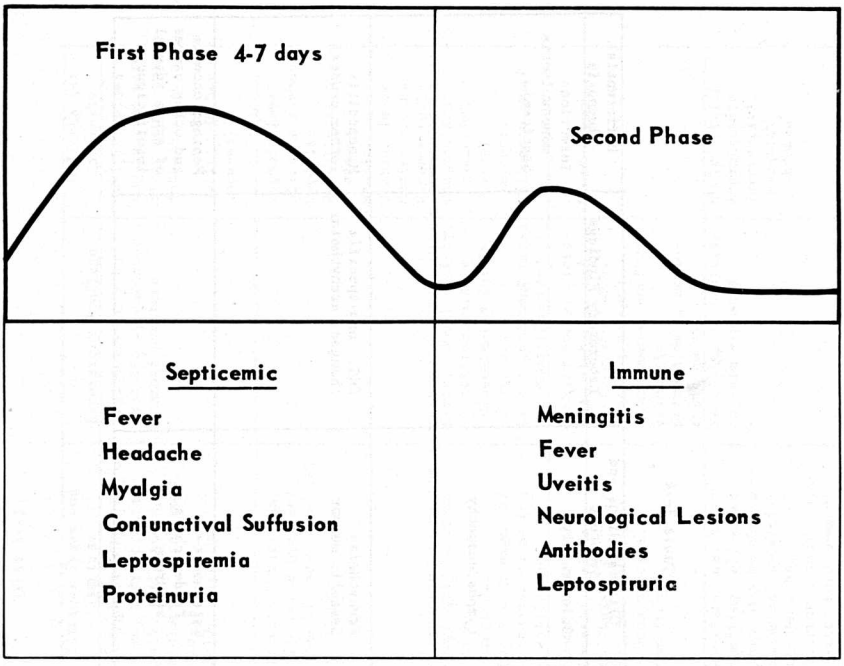
Table 3--4

| Organs Involved | Pathology | Clinical Signs and Symptoms | Laboratory Findings | Differential Diagnosis |
|---|---|---|---|---|
| Reticulo-endothelial system | Spleen: R-E hyperplasia throughout pulp, phagocytosis of fragmented RBC, depletion of lymphocytes.<br>Generalized enlargement<br>Lymph Nodes:/Lymphatic vessels dilated and contain histiocytes, RBC, and hemoglobin.<br>Proliferation of endothelial cells and RBC phagocytosis. | Splenomegaly<br>Lymphadenopathy | | Infectious mononucleosis<br>Septicemia |
| Heart | Interstitial myocarditis.<br>Foci of hyaline and granular degeneration.<br>Loss of striation in myocardial fibers.<br>Scattered foci of necrosis.<br>Mural ventricular endocarditis. | Arrhythmias<br>Systolic murmur | EKG: non-specific changes, arrhythmias | Myocarditis (other causes) |
| Adrenals | Hemorrhage<br>Interstitial inflammation with focal areas of necrosis (mainly in cortex) | Waterhouse-Friderichsen syndrome | | Meningococcemia and other causes of acute adrenal insufficiency. |

Figure 6

THE STAGES OF LEPTOSPIROSIS




From Edwards, Geo. A., and Domm, B. M. Human leptospirosis. Medicine 39:120, 1960

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The diagnosis should be suspected particularly in any case of febrile illness which presents with myalgia and conjunctival suffusion, and which is characterized by meningeal, renal, or hepatic involvement. Clinical suspicion should be further enhanced if there is present a history of environmental or occupational contact with wild or domestic animals, directly or indirectly (i.e., contaminated water). Although leptospirosis in its many different manifestations can mimic a wide range of other human diseases, the correct identification of the great majority of cases can be confirmed if such clinical and epidemiological awareness is exercised.

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## CLINICAL MANIFESTATIONS OF LEPTOSPIROSIS IN DOMESTIC ANIMALS

In animals, as in human beings, clinical signs and symptoms of leptospirosis vary greatly and differential diagnosis is essential. For example, leptospirosis in cattle may resemble brucellosis, vibriosis, anaplasmosis, bacillary hemoglobinuria, or other infections and physiological and toxic disorders.

The symptoms and signs of leptospirosis observed in cattle include abortion, usually during the seventh month of gestation, decreased milk production, abnormal lacteal secretion, hemoglobinuria, icterus, anorexia, fever, diarrhea, anemia, loss of weight, and general depression. The milk frequently becomes yellowish, thickened, and blood tinged, and the udder may be flaccid. In many herds, abortion may be the only sign. Deaths are observed to occur, but rates are quite low. Generalized infection within an entire herd over a period of time is most often seen. Since cattle usually have a fairly high serological titer and are asymptomatic at the time of abortion, it appears that abortion is not a manifestation of the acute phase of bovine leptospirosis. Retention of the fetal membranes following abortion occurs in some animals. The course of the disease is variable, with fever usually persisting for 3 to 5 days and other signs and symptoms, as chronic weight loss and anemia, for several weeks.

In the United States, most infections in swine are mild or asymptomatic. Abortions and the birth of weak pigs are the most obvious and sometimes the only evidence of infection in swine. Metritis and lowered fertility have been encountered occasionally, as well as icterus, anemia, fever, encephalitis, hyperirritability, and incoordination. Mortality in mature pigs is low, but morbidity generally includes most of the herd.

In horses, the disease is characterized by fever, depression or dullness, and anorexia. Icterus may be observed in some animals. Many cases of equine leptospirosis undoubtedly occur without being observed because most affected horses show only mild, transient symptoms. Clinical and serological evidence indicate that leptospirosis and periodic ophthalmia (recurrent iridocyclitis) in horses are closely associated.

The clinical manifestations in the dog are extremely variable and may be absent, latent, subclinical, atypical, mild, or severe. The symptoms and signs frequently observed include depression, anorexia, vomiting, fever early, or a subnormal temperature late. The sclera and conjunctiva are usually injected and small ulcers are frequently seen along the gums and oral mucosa. Tonsillitis and a peculiar foul breath are commonly reported. Icterus may or may not be a prominent symptom. Many animals are tucked up, having a tense abdominal wall and tenderness over the kidneys. A concurrent stiffness of the hindquarters may occur. Albuminuria, hematuria, and high blood urea nitrogen as the result of nephritis

are common laboratory findings. In acute infections, mortality is usually high and death occurs in 2 to 4 days unless treated.

Survey studies have shown that leptospiral infection of cats occurs rarely and usually fails to cause recognizable clinical manifestations of disease.

Sheep and goats are susceptible to experimental infection with leptospires, but natural infections are uncommon and only in isolated instances have outbreaks been reported in which severe clinical signs were observed. Abortion, hematuria, jaundice, and a high mortality rate were noted in both species.

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## Section II

## GENERAL LABORATORY METHODS IN DIAGNOSIS

Leptospirosis should be considered and laboratory diagnostic aid sought in every febrile illness of unknown origin, particularly if the patient's history indicates possible exposure to leptospires. Clinical manifestations which suggest aseptic meningitis, or nonparalytic poliomyelitis-like infections, also are caused frequently by leptospiral infections. Obviously, the procedures that will provide the earliest laboratory confirmation of the diagnosis of leptospirosis are the most desirable from the standpoint of patient, physician, veterinarian, and epidemiologist. These procedures, as in other bacterial infections, are based upon cultural and animal inoculation tests to isolate the leptospires, microscopic demonstration of leptospires in tissues or body fluids, and serological tests to detect leptospiral antibodies. The success and reliability of these laboratory tests depends largely upon their application at the proper time in the course of the disease and proper collection and handling of the sample to be examined.

### 1. ISOLATION PROCEDURES

#### A. Direct and Indirect Culture

Contrary to common beliefs, leptospires can be readily cultivated. The isolation of leptospires by direct culture from the blood of patients during the febrile stage of illness is a comparatively simple procedure and, if properly executed, is by far the most reliable. If a cerebrospinal fluid sample is collected during the first 10 days of illness, this should be cultured also. Leptospires are recovered more frequently from blood between the 2d and 8th days of illness but isolations have been made occasionally as late as the 19th day. Since the number of leptospires in the blood in human cases is usually very limited, several tubes (3 to 5) of a suitable semisolid medium should be inoculated. Inoculation of freshly drawn blood is most desirable, but leptospires reportedly will remain viable up to 11 days in blood to which anticoagulants have been added (*1, 2, 3*). (Sodium oxalate, 0.5 ml. of a 1-percent solution to 5 ml. of blood, or 1 ml. of a 1-percent solution of "liquoid" Roche in a sterile buffered saline solution at pH 7.5 to 5 ml. of blood.) Although citrate solutions are considered inhibitory by some investigators (*1*), Van Riel *et al.* (*3*) have isolated leptospires from blood of human patients, shipped in both the buffered "liquoid" and sodium citrate solution from

the Belgian Congo to their laboratory in Belgium. The samples were en route from 4 to 11 days.

Numerous media (*4, 5, 6, 7, 8, 9*) have been developed for the cultivation of leptospires but there has been compartively little change from the original medium prepared by Noguchi (*4*) in 1915 and used by Inada and Ido to isolate the first strains (*1*). Basically, these media usually consist of a buffer solution, with or without some type of peptone and with or without 0.1 to 0.2 percent agar, to which rabbit serum is added to provide a final concentration in the medium of 5 to 10 percent. The pH may range from 7.2 to 7.8. Other types of serum, including bovine, horse, goat, and human, have been used with varying degrees of success, but none of these have been found to be consistently superior to rabbit serum.

Since clinical materials available for culture are frequently contaminated, a selective medium would be of inestimable value for the isolation of leptospires. Sulfadiazine and sulfaguanidine, in low concentrations, were found to inhibit some contaminating micro-organisms (*7, 10*). In recent studies, several agents have been found in low concentrations that exhibit little or no effect on leptospires (*10, 11, 12*). Cycloheximide (actidione) has been used successfully to inhibit fungi. Encouraging results have been obtained, also, with vancomycin and bacitracin. Certainly, careful evaluation of these agents is indicated to determine their efficacy for use in routine isolation of leptospires.

The recommended proportion of patient's blood to medium varies from 1 ml. of blood to 3 ml. of medium (*13*) to 0.03 ml. of blood per 15 ml. of medium (*14*). In our experience, minimal amounts of inoculum have been more successful. Van Riel *et al.* (*3*) found that dilution of blood was of value. They inoculated 0.5 ml. of blood to 5 ml. of medium, then transferred 0.5 ml. of this mixture to a second tube containing 5 ml. of medium, and, finally, 0.5 ml. of mixture from the second tube to a third tube containing 5 ml. of medium.

Leptospires usually appear in the urine during the second week of the disease and may persist for 30 days or longer. Voided urine may be cultured directly if five to six serial dilutions are first prepared in sterile buffered saline or a suitable liquid leptospiral medium. If the sample is not diluted, overgrowth from contaminating organisms will occur. In such cases, inoculation into a test animal may be necessary. Weanling hamsters or guinea pigs are usually employed as test animals. More recently, gerbils (meriones) (*15*) and chinchillas (*16*) have been used successfully. Leptospires may be recovered also through 1- to 2-day old chicks (*17, 18*) but they appear to be slightly more refractory to small numbers of organisms than hamsters or guinea pigs. White mice have been used, but many laboratories have encountered natural infection with *L. ballum* in their mouse colonies (*19*). Natural infection in hamsters (*20*) and guinea pigs (*21*) must be considered, also. The

susceptibility of these animals varies with the leptospiral serotype, and they may or may not show clinical signs of infection. Attempts must be made, therefore, to isolate leptospires from their blood at periodic intervals after inoculation.

## 2. DEMONSTRATION METHODS

### A. Morphology of Leptospires

The morphologic appearance of leptospires is basically the same for all members of the genus *Leptospira*. They appear as slender thread-like organisms, about 0.1 micron in diameter and from 6 to 12 microns long, although they may be as long as 30 to 40 microns. The organism is tightly coiled on its long axis and the ends are usually bent like a hook, although on rare occasions the ends may be straight. The coils have an amplitude of 0.2 to 0.5 micron. Active young cultures rotate rapidly on their long axis. Electron micrographs have revealed an axial filament, through the entire length of the organism (*22, 23, 24*). They are highly motile and capable of passing Seitz E–K filters and other similar bacteriological filters. Leptospires cannnot be seen in wet preparations by light-field microscopy, but they can be seen easily with dark-field illumination. They stain poorly with the usual bacterial stains, but may be stained readily by several silver impregnation techniques. A convenient method for negative staining with Congo Red was reported recently by Hoyer (*25*).

**(1) Dark-field examinations.**—During leptospiremia, leptospires may be found by dark-field examination of the blood or they may be detected in the urine after the first week of the disease. This method so frequently results in failure or in misdiagnosis that it should never be relied upon as the only diagnostic test. Usually, so few organisms are present in one drop of blood that they may be missed, or artifacts such as protoplasmic extrusions from blood cells may confuse even experienced workers. Dark-field examination may be used also to demonstrate leptospires in the blood or body fluids of inoculated guinea pigs or hamsters. However, at best, it should be considered only as an ancillary method.

**(2) Silver staining.**—In fatal cases, leptospires may be found in sections of liver and kidney tissue stained by Levaditi's impregnation technique. Bridges (*26*) has reported recently on the satisfactory application of another silver staining procedure, Kerr's improved Warthin-Starry technique (*27*). Silver staining is recommended as a useful method of demonstration of leptospires in aborted bovine fetuses since isolation attempts from fetal material are frequently unsuccessful (*28*). It should be emphazised that no differentiation as to infecting serotype can be made by either of these demonstration techniques.

**(3) Fluorescent staining.**—Limited studies indicate that fluorescent antibody techniques may be applied successfully to the detection of leptospires in urine and tissue preparations (*29, 30, 31*). White and Ristic

(*29*) demonstrated *L. pomona* in the urine of experimentally infected calves by staining with fluorescent-labeled antibody. Recently, White *et al.* (*32*) successfully applied this technique to the detection of leptospires in naturally infected dogs. The technique may prove to be a valuable tool for detection of leptospires in the urine, particularly from carrier animals.

### 3. SEROLOGICAL PROCEDURES

Since isolation of leptospires from blood is usually possible only during the acute phase of the disease and, like animal inoculation, may require several weeks, laboratory diagnostic aid frequently is dependent upon serological demonstration of antibodies in the patient's serum. A variety of tests have been developed for the serodiagnosis of leptospirosis. Probably the most widely used method has been the microscopic agglutination test with live antigen (agglutination-"lysis" test). Although this test is time-consuming, hazardous, and the sensitivity of the living antigen is difficult to standardize, it still is considered the reference test for evaluation of other tests. The microscopic agglutination test with killed antigens (formalinized) is less hazardous and the antigens usually remain stable for at least two weeks. While titers are somewhat lower with killed antigens and cross reactivity is greater, results with the two tests compare favorably (*33*).

Within recent years, several macroscopic agglutination tests have been developed (*34, 35, 36*) which are performed easily and rapidly. They are of particular value in screening. The antigens are stable for at least 12 months and results have been highly satisfactory in comparative tests with microscopic agglutination methods. These tests tend to show serogroup specificity, which means that a battery of antigens or several pooled antigens should be employed. During the late acute phase of illness, however, a high degree of cross-agglutination is usually noted. More recently, a latex leptospiral tube agglutination test (*37, 38*) has been developed which is read macroscopically. These antigens are reported to be stable for 2 years.

Other tests include the sonic-vibrated leptospiral complement-fixation test (*39*), the erythrocyte sensitizing substance (ESS) test (*40*), and the hemolytic test (HL) (*41*). These tests tend to be somewhat genus specific and require fewer antigens to detect leptospiral antibody. They are more difficult to perform, however, and are not practical for use in small laboratories.

### 4. INTERPRETATION OF SEROLOGIC FINDINGS

In cases of leptospirosis, antibodies generally appear from the 6th to the 12th day of disease and increase rapidly, reaching maximum titers by the 3rd or 4th week. After infection, low agglutinin titers may persist for months or years. For this reason, it is frequently impossible to determine whether current leptospiral infection exists or antibodies are due

to past experience with the disease unless at least two blood samples are examined, one during the early stages of illness and another 10 days to 2 weeks later to detect a rise in titer. Serodiagnostic tests will not provide an early diagnosis, but, when carefully evaluated, together with clinical and epidemiological data, they are helpful in confirming active or past leptospiral infections.

Dependence should never be placed entirely upon serological reactions of the patient's serum to determine the infecting serotype. For example, it is frequently observed that during the acute phase of *L. icterohaemorrhagiae* infections paradoxical reactions may occur with other serotypes such as certain strains of *L. sejroe, L. andaman,* and others (*42, 43*). Kmety (*43*) has observed such reactions even with *L. biflexa.* Therefore, it is obvious that the infecting serotype can be determined with certainty only by isolation and serological identification of the leptospires. A negative reaction, even on serial samples, does not rule out the possibility of infection since the patient may be infected with a serotype not included in the battery of testing antigens. It should be pointed out, also, that failure to demonstrate a rise in titer may not eliminate the possibility of current infection. There is some evidence to indicate early antibiotic treatment may suppress the development of leptospiral antibodies so that they may appear late and show no increase or fail to appear entirely (*44*).

## RECOMMENDED PROCEDURES FOR LABORATORY DIAGNOSIS

### 1. ISOLATION PROCEDURES

#### A. Direct Culture

**(1) Blood.**—A blood sample should be collected aseptically during the first 10 days of illness before treatment with antibiotics and preferably when the patient is febrile. Swab the area with 70 percent alcohol before puncturing the vein, and use a sterile dry syringe and needle. Inoculate whole blood immediately into each of four tubes of Fletcher's semisolid medium (*8*) containing 5 ml. of medium per tube.[1] It is best to vary the number of drops of blood inoculated. Thus, inoculate one drop in one tube, two drops in the second, and three drops in the third and fourth tubes (fig. 7). Excessive amounts of blood may inhibit the growth of leptospires. If excessive amounts of blood are inoculated, an additional amount of medium should be added to the tube. The cultures are incubated at 28–30° C. (room temperature in the dark if a low temperature incubator is not available) for 5 to 6 weeks unless found to be positive earlier. In semisolid media, leptospires grow in a concentrated ring about 0.5 to 1 cm. below the surface. While this ring formation is helpful in detecting growth, it frequently does not develop for several weeks after inoculation

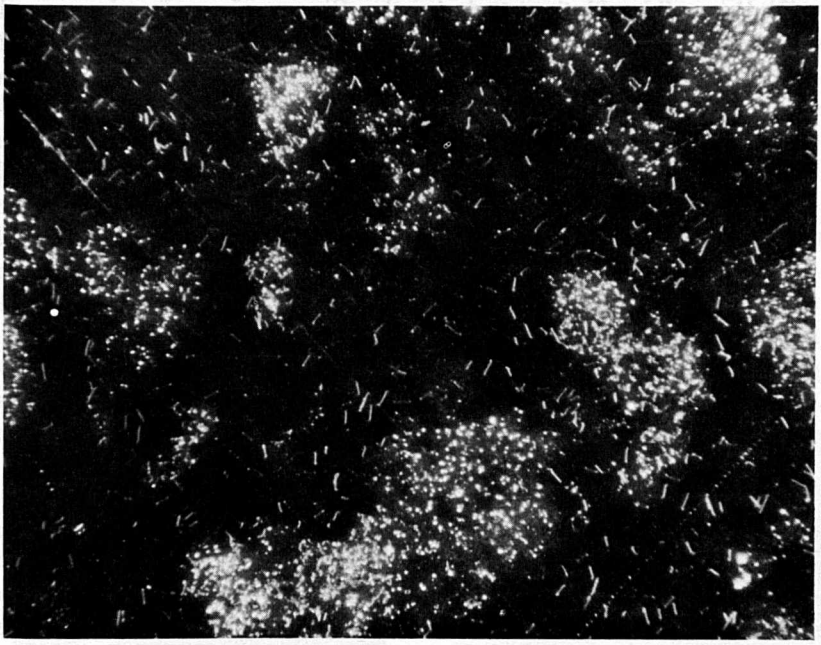
[1] If culture medium is not available, sodium oxalate may be added as indicated in I.A., p. 33, to prevent coagulation and the blood shipped to the laboratory.

**Figure 7. Inoculation of blood into Fletcher's medium.**

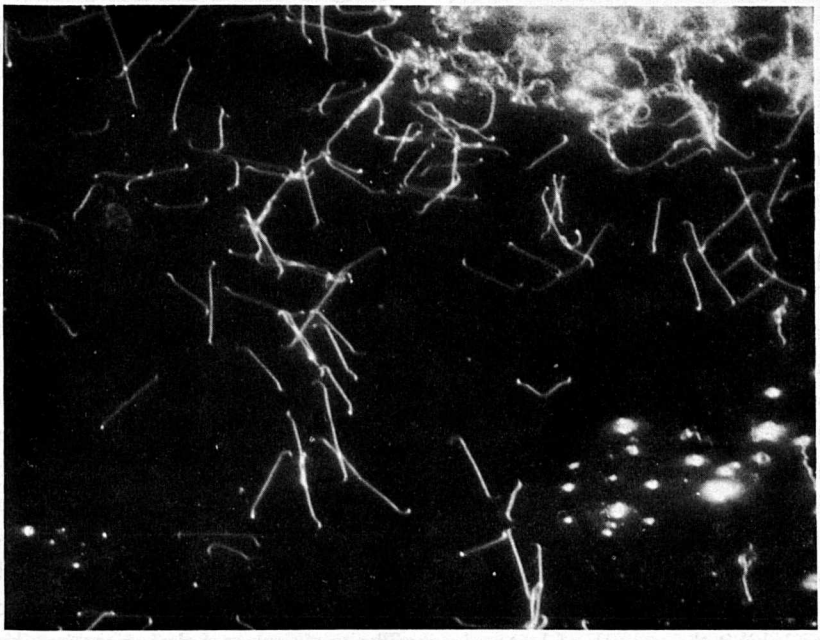
and occasionally not at all in the original culture. The cultures should therefore be examined at 7- to 10-day intervals microscopically. A large loopful of culture from the tube is placed on a 2- by 3-inch slide marked in ½-inch squares, without a coverslip. The drop is examined by dark-field illumination technique with a low-power objective 10× and 15× or 20× wide-field oculars. Suspicious or positive cultures should be rechecked with a coverslip on the drop, using 450× magnification. If a dark-field condenser is not available, a star diaphragm may be inserted in the Abbe condenser to obtain a dark background. It is important that a proper light source be used for dark-field work (fig. 8).

Contrary to general belief, this procedure is quite practical for hospital and public health laboratories. It involves the availability of a suitable semisolid medium, prompt inoculation of the patient's blood at the proper time, and a maximum of four examinations of the cultures. When protected against dehydration by screwcaps or vaccine-type rubber stoppers, Fletcher's medium may be used as long as 4 to 5 months after preparation. In public health laboratories, the medium may be provided to physicians or hospital laboratories on request, inoculated at the patient's bedside, and the cultures returned to the laboratory by mail (with precaution against freezing). This procedure has proved highly successful in the isolation of leptospires from the blood of infected dogs (*45*).

**(2) Cerebrospinal fluid.**—Spinal fluid, if collected during the first 10 days of illness, also may be cultured by the same method as blood. An inoculum of 0.5 ml. to 5–6 ml. of Fletcher's medium should be used (*46*).



a



b

**Figure 8. Photomicrograph of a leptospiral culture from blood in Fletcher's medium: a. Magnified approximately 150×; b. Magnified approximately 450×.**

**(3) Urine (by dilution technique).**—Direct culture has been used successfully to isolate leptospires from voided urine from both man and animals if the sample is first diluted in buffered saline (*47*). With a sterile dry 2 ml. syringe and a 1-inch, 20-gauge needle, one drop of undiluted urine is inoculated into a tube of Fletcher's medium. Then, with the same syringe, dilutions of the urine are prepared with sterile buffered saline as follows: Discharge all but 0.1 ml. of the undiluted urine from the syringe and draw 0.9 ml. of buffered saline into the syringe. Expel a few drops of this 1-in-10 dilution of urine and inoculate one drop into a tube containing 5 ml. of Fletcher's medium. Continue this procedure for four additional dilutions. Stuart's base medium or simply a 0.2-percent peptone broth may be used also as a diluent. Bacterial contaminants usually appear in the undiluted urine cultures but leptospires in pure culture have been obtained in cultures of the same sample when diluted.

**(4) Urine (by bladder tap).**—Recently, a technique has been developed whereby urine suitable for direct culture may be obtained from animals by puncture of the bladder through the abdominal wall (*48*) (figs. 9a, b, c, d). This procedure has been used successfully on dogs, cats, guinea pigs, and small wild mammals, and it should be equally valuable for use on sheep, swine, goats, and young calves. Successful application of needle aspiration of the bladder in human patients was recently reported by Monzon *et al.* (*49*) in a comparative study of bacterial counts of urine obtained by this method, catheterization and midstream-voided methods. Their results revealed a greater number of sterile specimens were obtained by suprapubic aspiration of the urinary bladder without undesirable side effects.

The animal is best held by its hindlegs with the head down, or it may be kept in a standing position. Swab the abdomen with 70 percent alcohol. With a 10 ml. sterile dry syringe and a 3-inch, 20-gauge needle, aspirate about 5 ml. of urine by entering the bladder along the midline and just anterior to the brim of the pelvis. For small dogs, a 1-inch, 20-gauge needle should be used. Inoculate one drop of urine immediately into each of two tubes of Fletcher's semisolid medium containing 5 to 6 ml. of medium per tube. Then prepare two dilutions of the urine with sterile buffered saline solution or Stuart's base medium beginning with 1 in 10, as described in (3) above, and inoculate one drop of each dilution into each of two tubes of medium. Studies on experimentally infected guinea pigs and field studies on animals have indicated that excessive amounts of urine appear to inhibit the growth of leptospires. The remainder of the urine may be examined by agglutination tests to determine the presence of urinary antibodies, if desired.

This technique is very useful in determining the source of human infection, particularly if the family pet is suspected. Serologic tests will give

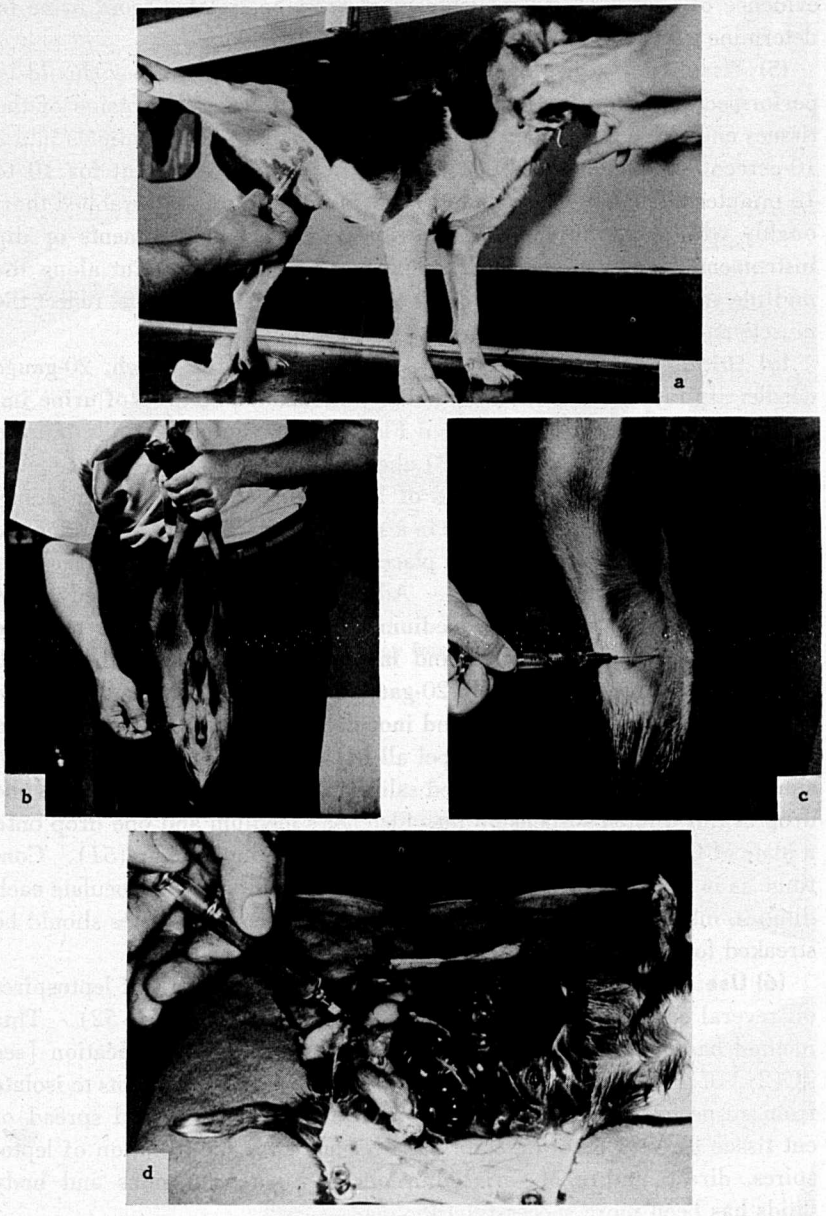



**Figure 9. Obtaining urine by bladder tap. a. From a male dog in standing position; b. From male dog in a suspended position; c. From a female dog in a suspended position; d. From a guinea pig at necropsy.**

evidence of leptospirosis, but leptospires must be isolated from urine to determine whether an animal is a carrier.

**(5) Tissue and urine cultures at necropsy.**—The necropsy should be performed as soon after death as possible since bacterial invasion of the tissues and urine may occur. Prior to necropsy, dip small animals into a 10-percent saponated cresol solution or a similar disinfectant for 10 to 15 minutes. Human cadavers and large animals should be swabbed thoroughly with the solution. Use previously sterilized instruments or dip instruments in 95 percent alcohol and flame to sterilize. Cut along the midline and remove the skin. With another set of instruments, reflect the muscle and peritoneum to expose the viscera.

**(a) Urine.**—With a 2 ml. sterile dry syringe and a 1-inch, 20-gauge needle, aspirate urine from the bladder. Inoculate one drop of urine immediately into each of two tubes of Fletcher's medium, then dilute urine and inoculate as described in 1A(3) above.

**(b) Kidney.**—Remove portions of both kidneys or the entire kidney of small animals. Place the tissue in a sterile Petri dish, and determine the weight in grams (fig. 10). Then place the tissue in a sterile mortar with sterile sand and grind (fig. 11). Add 9 ml. of sterile buffered saline (pH 7.2) solution or Stuart's medium for each gram of tissue to make a 10-percent tissue suspension and mix well (fig. 12). With a 2 ml. sterile dry syringe and a 1-inch, 20-gauge needle, draw off about 1 ml. of the kidney suspension (fig. 13) and inoculate three drops into each of two tubes of Fletcher's medium. Expel all but 0.1 ml. of the kidney suspension, then draw 0.9 ml. of buffered saline into the syringe. Inoculate one drop of this diluted suspension into Fletcher's medium and one drop onto a plate of Cox's solid medium (*50*) or modified Stuart's agar (*51*). Continue as in 1A(3) to prepare three additional dilutions and inoculate each dilution into semisolid and solid medium as above. The plates should be streaked for isolation with a sterile loop or glass spreader.

**(6) Use of agar plate technique.**—The colonial growth of leptospires on several solid media has recently been described (*50, 51, 52*). This method has numerous advantages for isolation and for purification [see 1C(2)] of leptospires, but it should not be used alone in attempts to isolate from suspected infected material. Although impression and spread of cut tissue directly onto the plate has been used for the isolation of leptospires, direct culture of serial dilutions of tissue emulsions and body fluids has been more successful (*45, 53*).

Material to be cultured should be diluted serially tenfold. For most materials, isolated colonies of leptospires may be obtained at from $10^{-3}$ to $10^{-6}$ dilutions. Very heavily contaminated materials should be plated in higher dilutions. A single inoculating loopful (4 to 5 mm. in diameter) of each dilution should be placed at one edge of the agar and spread over the entire surface of the plate with the loop or a glass spreader. The inoculated plates should be incubated at 30° C. overnight in an upright

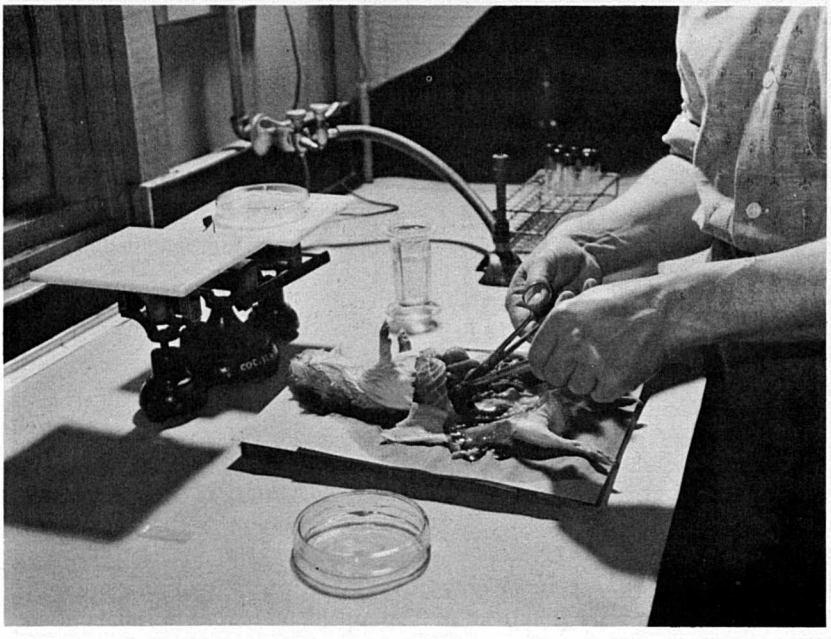


**Figure 10. Removal of kidney from a guinea pig at necropsy.**

**Figure 11. Placing kidney in sterile mortar with sterile sand and grinding.**

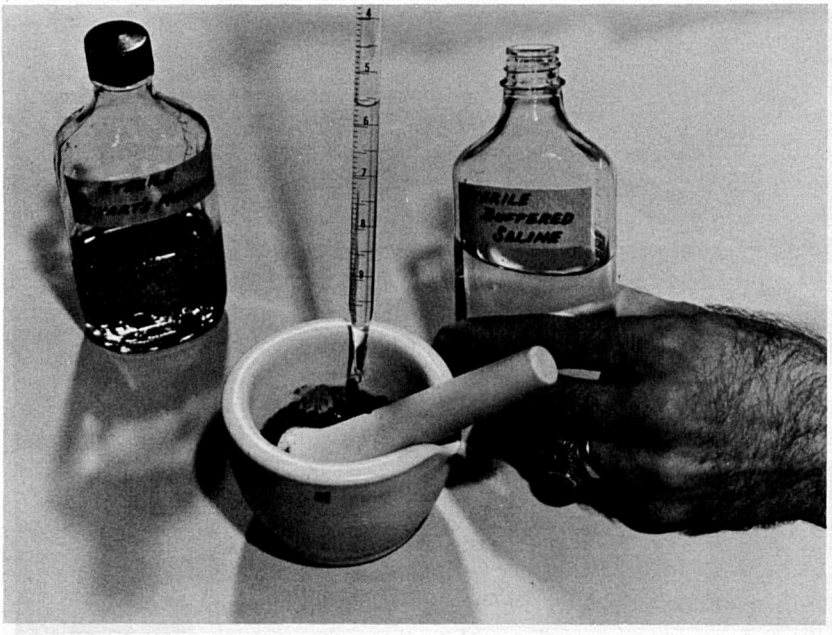



**Figure 12. Addition of diluent, sterile buffered saline, or a liquid leptospiral medium to ground kidney.**

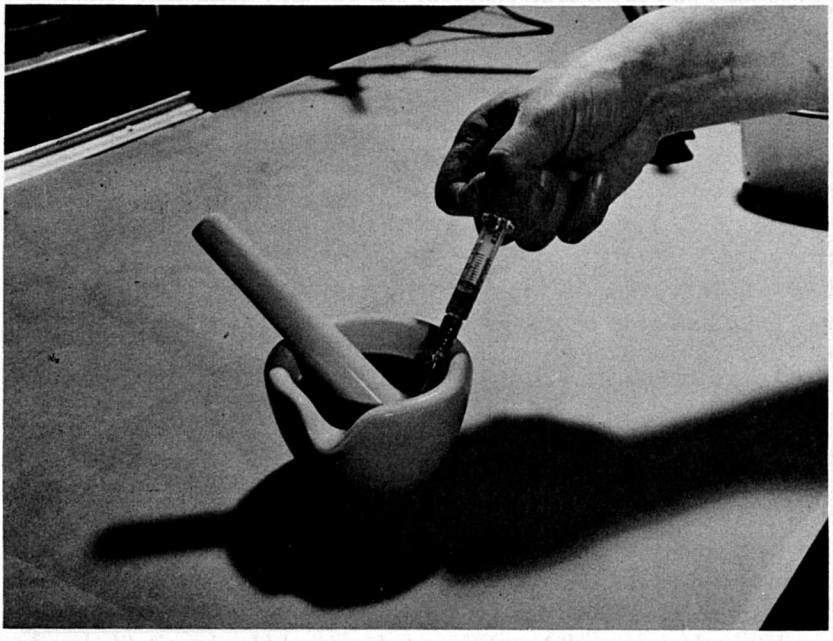


**Figure 13. Filling syringe with kidney suspension to inoculate culture medium, and prepare further dilutions.**

position to allow surplus moisture to evaporate. They should then be sealed with either adhesive or "masking" tape and reincubated at 30° C. in an inverted position for 1 to 8 weeks and observed periodically for growth.

Colonies usually appear subsurface and spread slowly. They are bluish-gray-white. In low dilutions where many viable organisms are present, individual colonies may not be formed but leptospires appear as a bluish gray, subsurface haze covering most of the plate.

### B. Animal Inoculation

The animals used include hamsters, guinea pigs, chicks, and gerbils. Hamsters appear to be the animal of choice. The same animal inoculation techniques may be used to isolate leptospires from blood, spinal fluid, or tissue if the specimen is suspected to be contaminated.

**(1) Hamsters.**—Three 21-day-old hamsters are each inoculated intraperitoneally with 0.5-ml amounts of urine. Each hamster is anesthetized and bled from the heart, using a sterile, dry, 2 ml. syringe with a ¾-inch, 25-gauge needle. The best bleeding period is from the 5th to 10th day after inoculation. Four bleedings on the 5th, 8th, 10th, and 14th days postinoculation should be adequate. Immediately after blood is obtained, inoculate 1 to 3 drops into each of four tubes of Fletcher's semisolid medium. *Excessive amounts* of blood should *not* be inoculated. The animals should be held for 21 days, then sacrificed, the kidneys removed, and a suspension prepared for direct cultures as in (5) (b). The same technique is used for gerbils (meriones).

**(2) Guinea pigs.**—Use three weanling (4-week-old) animals of approximately 200 grams. Inoculate 0.5- to 1-ml. amounts of urine intraperitoneally. Take rectal temperatures from the 3rd to 10th days. Bleed animals from the heart when they show fever (104° F. or greater). However, since some animals may not show a rise in temperature, the animals should be bled also at periodic intervals as with hamsters. On the 28th day, the animals can be necropsied and urine and kidney tissue cultured.

**(3) Chicks.**—Inoculate three 1-day-old chicks each intraperitoneally with 0.5-ml amounts of urine. Bleed the chicks with a 25-gauge needle from the heart on the 3rd or 4th, 10th, 15th, 18th, and 25th days postinoculation. As the chick gets larger, 20- or 22-gauge, 1-inch needles may be used.

### C. Purification of Contaminated Cultures

If, on initial isolation, leptospiral cultures are contaminated or if stock cultures become contaminated, they may be purified by several methods:

(1) If there are numerous leptospires in the culture, it may be purified by filtration through a Swinny filter with an ST–3 filter pad and inoculation of the filtrate directly into Fletcher's or Stuart's media. This method is not usually successful when only a few leptospires are present in the culture.

(2) Dilution of contaminated culture and direct inoculation of each dilution onto ia plate of solid medium. Prepare ten fold dilutions of the contaminated culture in sterile buffered saline or in sterile Stuart's medium. Inoculate 0.1 ml. of each dilution $10^{-3}$ through $10^{-7}$ onto a Cox agar plate, or Stuart's medium with 1 percent agar added and streak for isolation.

On the solid medium, leptospiral colonies will be observed as subsurface colonies which may be picked quite easily by blocking out an area with a sterile scalpel and transfer of the block to a tube of semisolid leptospiral medium, or a plug may be obtained with a sterile capillary pipette for subculture.

(3) The contaminated culture may be inoculated intraperitoneally in the amount of 0.5 to 1 ml. into a hamster or guinea pig. After 10 to 15 minutes, the animal is bled from the heart and the blood inoculated into several tubes of semisolid medium (1 to 3 drops of blood per 5 ml. of medium).

### D. Maintenance of Cultures

Since methods to preserve leptospires by freezing or lyophilization have not produced consistently satisfactory results, cultures should be maintained by periodic transfer in Fletcher's semisolid medium. This medium should contain rabbit serum and should not be used until it has been checked for ability to support growth of leptospires.

All stock strains and new isolations are subcultured to at least two tubes of Fletcher's medium upon receipt in the laboratory. An inoculum of 0.5 ml. per 5 ml. of medium is adequate. Subsequent transfers to maintain the strain are made at 3-month intervals. If possible, two persons should each maintain a set of the stock strains independently. After inoculation, the cultures are incubated at 30° C. for 2 to 3 weeks, then checked macroscopically for growth. Usually, within this period, a ring of growth will appear one-half to 1 cm. below the surface of the medium. At this time, they may be placed in the dark at room temperature provided temperature of the storage area does not fluctuate too greatly (approximately 20°–25° C.).

It is desirable to retain at least four or five previously transferred reference sets in storage. If, for any reason, it is necessary to transfer from the current set of reference cultures, duplicate transfers should be made and one of these used to replace the transferred culture. Under *no* circumstance should a reference culture which has been opened be returned to the reference set.

*Extreme caution* should be taken to maintain accurate records, to eliminate possible errors in labeling tubes, and to prevent contamination.

## 2. SEROLOGICAL PROCEDURES

A rapid macroscopic slide agglutination test (*36*) is used to screen human and animal serum samples. If this test is positive, the titer

is determined by the microscopic agglutination test with live or formalinized antigens. The 12 leptospiral strains used to prepare antigens for both tests are representatives of each serogroup in which there is cultural or serologic evidence to indicate the presence of some member of the group in the United States. They include the following serotypes: *L. ballum*, S–102, *L. canicola*, Ruebush, *L. icterohaemorrhagiae* AB, Wijnberg, *L. bataviae*, Van Tienen, *L. grippotyphosa*, Andaman, *L. pyrogenes*, Salinem, *L. autumnalis*, AB Akiyami A, *L. pomona*, Johnson, *L. sejroe*, Mallersdorf, *L. australis*, Ballico, *L. hyos* hyos, and a recently isolated subserotype of the hebdomadis group, *L. mini georgia* (LT117). Serotypes representative of other serogroups are added to the battery for the microscopic test as desired.

Cross-agglutination studies with 17 slide test antigens and antiserums against 68 leptospiral serotypes or subserotypes indicated that, with most of the antiserums, some degree of cross-reactivity occurred with one or more of the battery of 12 antigens. *L. cynopteri* and *L. celledoni* antiserums failed to cross with any of the 12 antigens, but serums from two of three patients infected [1] with *L. celledoni* were positive in *L. canicola* and *L. javanica* antigens. Thus, in certain areas of the world, it may be desirable to include *L. cynopteri*, *L. celledoni*, and *L. javanica*. The only other exceptions were *L. semerang* and *L. andaman;* however, there is ample evidence which indicates that these two serotypes are more closely related to the saprophytic leptospires than to the pathogenic types.

### A. Macroscopic Slide Agglutination Test

**(1) Preparation of antigens.**—The leptospires are grown in Stuart's medium, dispensed in 500-ml. amounts in 32-ounce screwcapped prescription bottles. Each bottle is inoculated with 50 ml. (1:10 ratio) of an actively growing 4- to 6-day-old leptospiral culture. The inoculated bottles are incubated in a slanted position at 28°–30° C. for 5 to 6 days, then the cultures are examined by dark-field microscopy for density, autoagglutination, and contamination. A satisfactory antigen should contain at least 100 to 200 organisms per high power field (10× oculars and 45× objective), be free of "breeding nests," and free of contamination. If the cultures appear satisfactory, formalin is added to a final concentration of 0.5 percent and they are allowed to stand for 1 to 2 hours, or, if desired, they may stand overnight.

The killed antigens are then centrifuged in 50 ml. tubes for 10 minutes at 1,500 times gravity to remove extraneous material. They are checked again by dark-field microscopy for autoagglutination, and only smooth, nonclumping cultures are used. The supernatant is transferred to 50 ml. plastic tubes and centrifuged in a high speed, angle head, SS–1 Servall

[1] Serum from patients in Malaya made available by Dr. A. D. Alexander, Walter Reed Army Institute of Research.

or similar centrifuge for 25 minutes at 5,000 times gravity to pack the leptospiral cells. The supernatant is carefully poured off immediately and the tubes allowed to drain in a slanted position.

The sedimented cells from 40 ml. of culture are resuspended in 1.5 to 2 ml. of a sterile solution containing 0.5 percent formalin, 12 percent sodium chloride, and 20 percent glycerine. The antigen is mixed 15 to 20 times using a 2 ml. Cornwall pipette with a No. 16- to 18-gauge needle and pooled in 6- to 8-ounce prescription bottles for further processing. If the cells fail to resuspend completely as determined by dark-field microscopy, the clumps are removed by centrifugation for 10 minutes at 1,500 times gravity.

To standardize for density, the antigens are adjusted to 25 to 27 percent light transmission by the Lumetron photoelectric colorimeter with a No. M550 filter and a No. 6 reduction plate. Other equipment of comparable performance could be standardized against aliquots of the antigen and used. The Diagnostic Reagents Section, Communicable Disease Center (*54*) recommends for standardization of density, an alternate method in which international units are determined according to the "International Reference Preparation for Opacity" (*55*). This international reference preparation to which an opacity of 10 international units have been assigned may be obtained from:

1. Biologics Control Laboratories
   National Institutes of Health
   Bethesda, Md.
2. National Laboratories for Biological Standards
   Department of Biological Standards
   Statens Seruminstitut
   Copenhagen, Denmark

The preparation is a suspension of small particles of Pyrex glass in distilled water containing 1 part merthiolate per 10,000. It is stable for several years.

By this method, leptospiral antigens are standardized to 13 to 15 international units, which has been found to be approximately equivalent to 25 to 27 percent light transmission by the Lumetron photoelectric colorimeter or to 45 to 48 percent light transmission by the Coleman, Junior, instrument. Other equipment of comparable performance could be standardized against aliquots of the antigen and used. The final yield of antigen per liter of culture after standardization varies from 5 to 10 percent. The standardized antigens are then tested for sensitivity against the homologous rabbit antiserums and against the 11 heterologous rabbit antiserums for cross-reactivity. The antigens should also be tested against known positive human and animal serums of varying titer and negative serums.

**(2) Slide test procedure.**—(*a*) For screening, the 12 slide antigens are combined into 4 pools by mixing equal portions of three antigens per pool, as follows:

| *Pool I* | *Pool II* | *Pool III* | *Pool IV* |
|---|---|---|---|
| *L. ballum* | *L. bataviae* | *L. autumnalis* | *L. australis* |
| *L. canicola* | *L. gripptoyphosa* | *L. pomona* | *L. hyos* |
| *L. icterohaemorrhagiae* | *L. pyrogenes* | *L. sejroe* | *L. mini georgia* |

These antigens should be pooled in small portions, depending upon the volume of anticipated samples to be tested, so that fresh pools may be prepared every 6 to 8 weeks. The use of pooled antigens alone without confirmation with the single antigens is not recommended. However, if this is necessary, the serum should be sent to a reference laboratory for titration by either this test or the microscopic agglutination test with live antigens. A fifth pool containing *L. cynopteri*, *L. celledoni*, and *L. javanica* would be an advantage for certain areas of the world.

(*b*) For each pooled antigen used, place 0.01 ml. of serum to be tested on the glass plate (8- by 15-inch plate etched in 1-inch squares) with a 0.2-ml. pipette. Add one drop of antigen (approximately 0.055 ml.) and mix thoroughly with applicator stick or toothpick, then rotate plate by hand five to six times (figs. 14 and 15). Place plate on mechanical rotator with speed regulator set for 125 r.p.m. for 4 minutes (fig. 16).

(*c*) Remove and read reaction (fig. 17) over a light box or any good indirect light with black background. Agglutination is recorded as positive (+) when definite clumping occurs doubtful (±) if only slightly clumped, and negative (−) if the serum-antigen mixture is evenly suspended.

(*d*) If a positive or doubtful reaction is obtained in one or more of the pooled antigens, the serum is then tested with the single antigens of the respective pools. For this test, the same procedure described in (2) (*b*) is used. In reading, degree of agglutination is recorded as 4+ when all organisms appear clumped and the supernatant is clear; 3+ if approximately 75 percent of the leptospires have agglutinated; 2+ with 50 percent agglutination; and 1+ with 25 percent. Absence of agglutination characterized by an even suspension of serum-antigen mixture is recorded as negative (fig. 18). The end point is taken as the last dilution showing a 1+ reaction.

(*e*) If a positive reaction is obtained in one or more antigens, the serum is diluted 1:5 and titrated against those antigens. For titrations, the diluted serum (1:5) is placed on the glass plate in 0.04-, 0.02-, 0.01-, and 0.005-ml. amounts. A drop of the desired antigen is placed on each drop of diluted serum, mixed, rotated, and read as above.

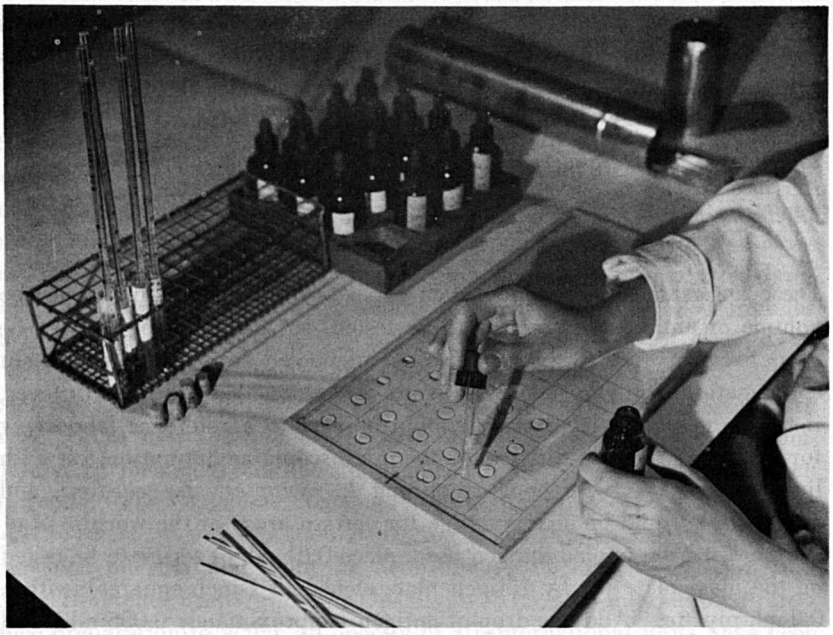


Figure 14. Setting up macroscopic slide agglutination test.

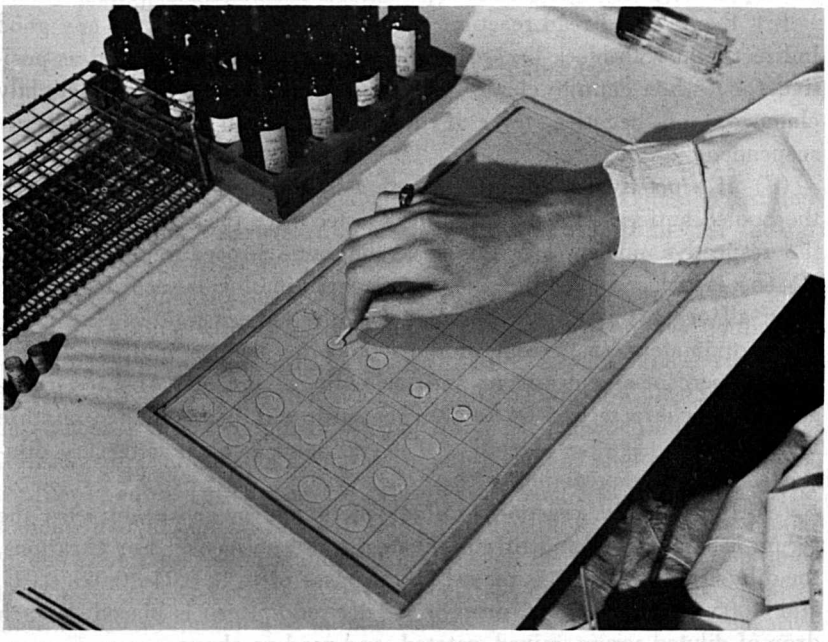


Figure 15. Mixing serum and antigen in the slide agglutination test.

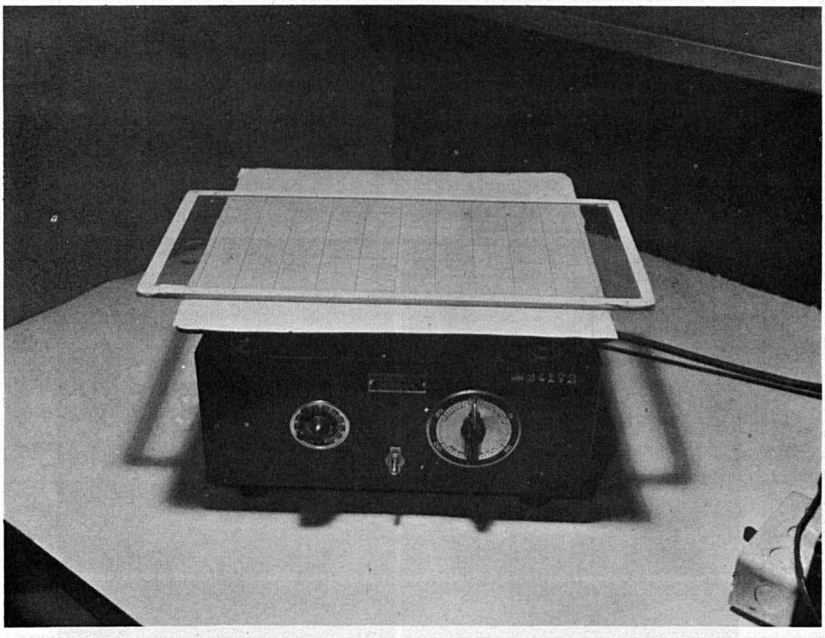


Figure 16. Rotation of serium-antigen mixture for slide agglutination test.

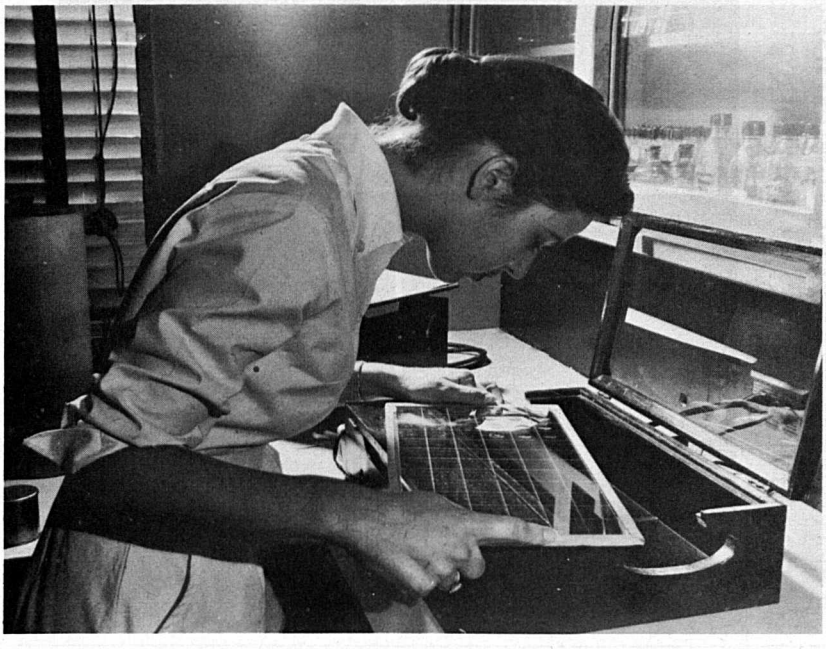


Figure 17. Reading macroscopic slide agglutination test.

LEPTOSPIRAL MACROSCOPIC SLIDE AGGLUTINATION TEST SHOWING NEGATIVE REACTION AND DEGREES OF POSITIVE REACTIONS

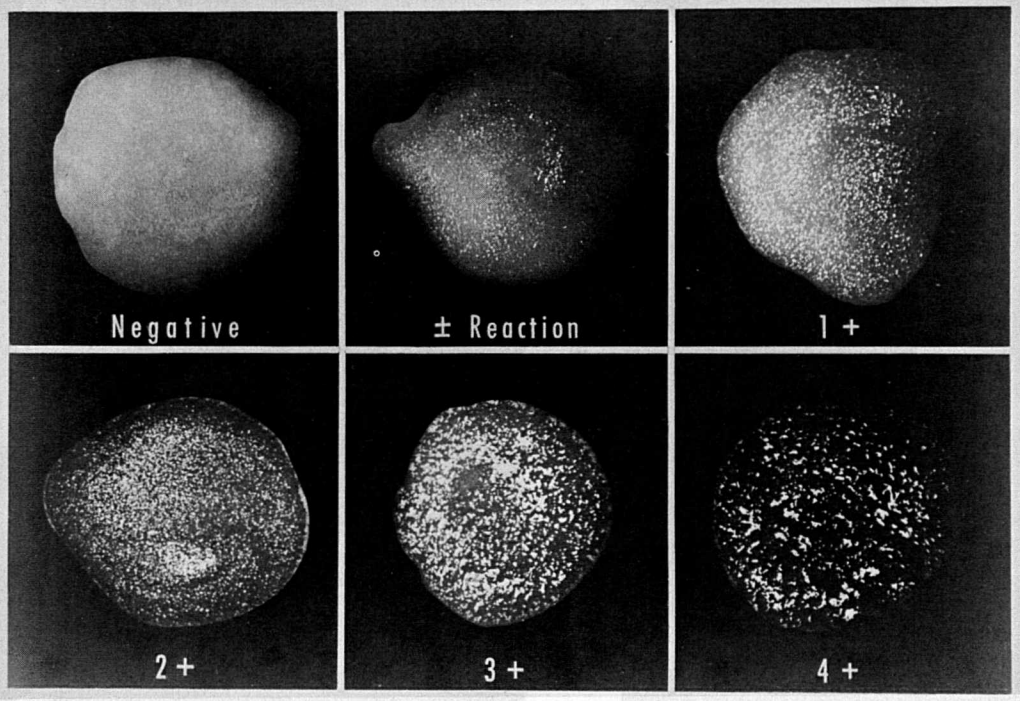



Figure 18

(*f*) If an end point is not reached and it is desired, the serum may be diluted 1:50 and titrated further. For this step, place the diluted serum (1:50) on the plate in 0.04-, 0.02-, 0.01-, and 0.005-ml. amounts and add one drop of the desired antigen to each drop of diluted serum, mix, rotate, and read as described in (2) (*d*).

In comparative studies with the microscopic agglutination test with formalinized antigen and the macroscopic slide test, it was found that for each microscopic test titer of 1:512 or above, the slide test median titer (*36*) was 5 (i.e., positive in the fifth dilution). It was found, further, that the slide test median titer increased gradually with the microscopic test titer. Thus, the slide test titer will serve as a guide to the approximate microscopic test titer and as to the number of dilutions necessary to set up if a microscopic titer is desired. The protocol for titration by slide test is shown on page 54.

During the past 3 years, serum samples received from more than 10 suspected human cases of leptospirosis have appeared positive by the macroscopic slide agglutination test but failed to show a reaction with any of the 12 antigens in the microscopic agglutination test with live antigens. These initial samples were collected within 4 to 7 days after onset of illness. However, second serum samples requested and received 10 days to 2 weeks later were positive by both tests in every instance. Due to the greater cross-reactivity, especially during the acute phase of illness, with killed slide test antigens than with the live antigens, the slide test is considered a more reliable screening procedure.

**Reporting.**—Reactions should be reported according to antigen, degree of agglutination and dilution. For example, serum reacted with *L. canicola* antigen, 1/7; *L. ballum*, 1/8; *L. icterohaemorrhagiae*, 1/6, or if paired serums are examined:

| Antigen | Serum No. 1 reaction | | Serum No. 2 reaction | |
|---|---|---|---|---|
| | Slide | Microscopic | Slide | Microscopic |
| *L. ballum* | [1] 1/7 | 1:50 | 1/8 | 1:100 |
| *L. canicola* | ±/1 | 1:50 | 1/7 | 1:400 |
| *L. icterohaemorrhagiae* | 1/3 | — | 1/6 | 1:200 |
| *L. bataviae* | 1/2 | — | 1/4 | 1:100 |
| *L. grippotyphosa* | 1/2 | — | 1/4 | 1:100 |
| *L. pyrogenes* | 1/2 | — | 1/4 | 1:50 |
| *L. autumnalis* | 1/3 | 1:100 | 1/4 | 1:200 |
| *L. pomona* | 1/3 | — | 1/4 | — |
| *L. sejroe* | 1/2 | — | 1/4 | — |
| *L. australis* | 1/4 | — | 1/4 | — |
| *L. hyos* | [2] — | — | 1/2 | — |
| *L. mini georgia* | — | — | 1/3 | — |

[1] The degree of agglutination is indicated in the numerator and the dilution number in the denominator.

[2] — = negative in initial dilution of slide test, and negative in 1:50 dilution of microscopic test.

## Protocol for Titration by Slide Test

| Dilution No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| L | undil. | diluted 1:5 | | | | diluted 1:50 | | | |
| Serum(ml) | 0. 01 | 0. 04 | 0. 02 | 0. 01 | 0. 005 | 0. 04 | 0. 02 | 0. 01 | 0. 005 |
| Antigen(ml) | 0. 055 | 0. 055 | 0. 055 | 0. 055 | 0. 055 | 0. 055 | 0. 055 | 0. 055 | 0. 055 |
| Actual dilution | 1:6. 5 | 1:11. 9 | 1:18. 8 | 1:32. 5 | 1:60 | 1:119 | 1:188 | 1:325 | 1:600 |

Dilutions in which reactions may be expected by microscopic test with formalized antigen.

1:16

1:32

1:64

1:128

1:256

1:512>

## B. Microscopic Agglutination Test

**(1) Preparation of Antigens.**—The 12 serotypes referred to under 2, page 47, are used routinely. However, if paired serum samples appear positive by the slide test and negative by the microscopic test with these 12 antigens, a second battery of antigens should be used also. These include *L. javanica, L. cynopteri, L. sentot, L. djasiman, L. alexi, L. borincana,* and *L. celledoni.*

Leptospiral strains used for antigen production are maintained in Stuart's medium and transferred every 5 days. The rapid transfer appears to keep the formation of "breeding nests" at a minimum. Such actively growing seed cultures are used in approximately 1:10 ratio to inoculate the desired amount of Stuarts' medium in screwcapped prescription bottles. The inoculated bottles are incubated 4 to 6 days at 28°–30° C. in a slanted position and then examined by dark-field microscopy for density, smoothness, and purity. If the antigens appear too dense, they may be diluted with Stuart's medium or buffered 0.85 percent saline solution. Very dense antigens will be undersensitive, but, if very light, they tend to be too sensitive and titers will be higher (*56*). A desirable antigen should contain 100 to 200 organisms per high-power field (10× oculars and 45× objective). Some cultures will develop small clumps of leptospires, however, frequent, successive transfer tends to eliminate development of these aggregates. Contaminated cultures should not be used. Sensitivity of each lot of antigen should be checked against the homologous antiserum before use. A satisfactory antigen should react within a twofold dilution above or below the known titer of the antiserum. If a fourfold or greater difference in titer exists, it should not be used. If live antigens are to be used, they are ready for addition to the diluted serum. If killed antigens are to be used, 0.3 percent formalin is added to the cultures and they are allowed to stand 1 to 2 hours, then centrifugalized for 10 minutes at 1,500 times gravity to remove extraneous material, then tested for sensitivity against homologous antiserums. These formalinized antigens usually remain stable for 1 to 2 weeks. They should be checked with positive and negative control serums prior to use and watched closely for spontaneous agglutination. Although comparable, titers with the formalinized antigens usually appear somewhat lower than titers with live antigens. In addition, wider cross-reactions with heterologous serotypes occur with the killed antigens.

**(2) Agglutination test procedure with live antigens.**—Serial twofold dilutions of serum are prepared with buffered 0.85 percent saline to provide dilutions of 1:25 through 1:3,200 (higher, if necessary, to reach end point). To 0.2 ml. of each serum dilution, 0.2 ml. of antigen is added (fig. 19). The tubes are shaken, incubated at 30° C. for 3 hours, and examined. A drop from each tube is examined by dark-field microscopy using low-power objective and 10× or 15× oculars without a coverslip (fig. 20). The degree of agglutination or "lysis," or both, is read as 1+,

**Figure 19. Setting up microscopic agglutination test.**

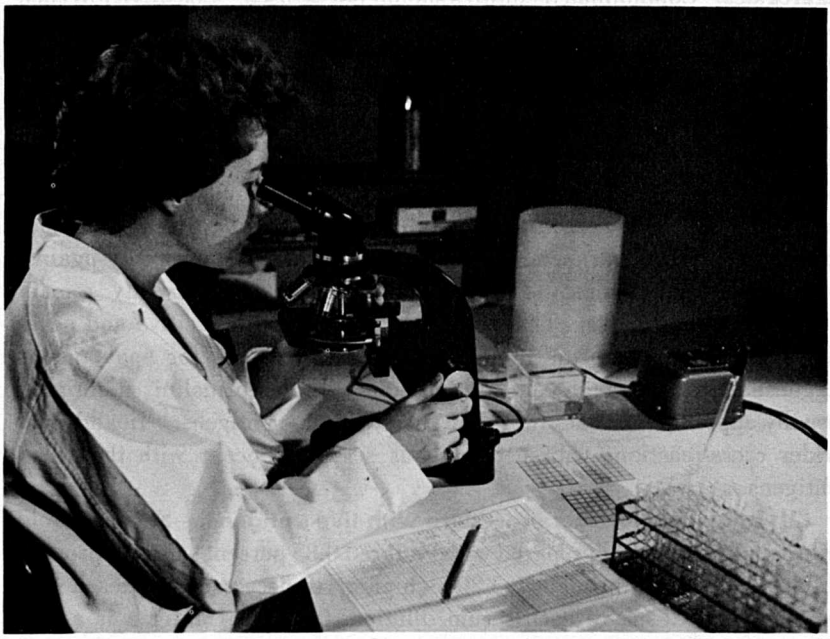


**Figure 20. Reading microscopic agglutination test.**

2+, 3+, 4+, or negative (figs. 21 a, b, c, d, e; 22 a, b, c, d, e). A reaction is recorded as 4+ when 75 to 100 percent of the leptospires appear clumped, 3+ when approximately 75 percent of the organisms are agglutinated, 2+ with about 50 percent agglutinated, and 1+ with at least 25 percent agglutinated. The end point is taken as the last dilution showing a 2+ reaction.

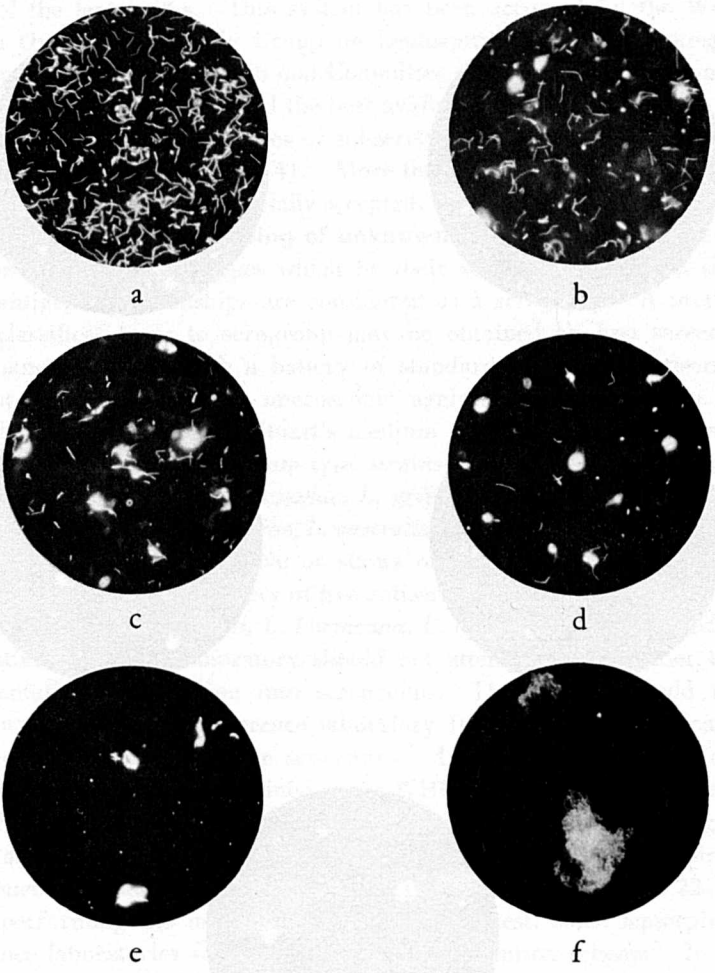



**Figure 21. Microscopic agglutination with live *Leptospira hyos* antigen: a. negative; b. 1+ reaction; c. 2+ reaction; d. 3+ reaction; e. 4+ reaction; f. microscopic agglutination with formalinized *L. hyos* antigen, 4+ reaction.**

**(3) Agglutination test procedure with formalinized antigens.**— Serial twofold dilutions of serum are prepared in buffered 0.85 percent saline, starting with 1:12.5 through 1:3,200 (higher, if necessary) in a final volume of 0.2 ml. To each serum dilution, 0.2 ml. of antigen is added (fig. 19). The tubes are shaken, incubated in a waterbath at

52° C. for 2 hours, and then refrigerated for 1 hour. Drops of the respective dilutions are placed on a slide and agglutination read as described above. It should be pointed out that, with formalin-killed antigens, the appearance of agglutination is different from that with live antigens. The clumps tend to be larger, and less tightly packed, giving a lacy appearance (fig. 21f).

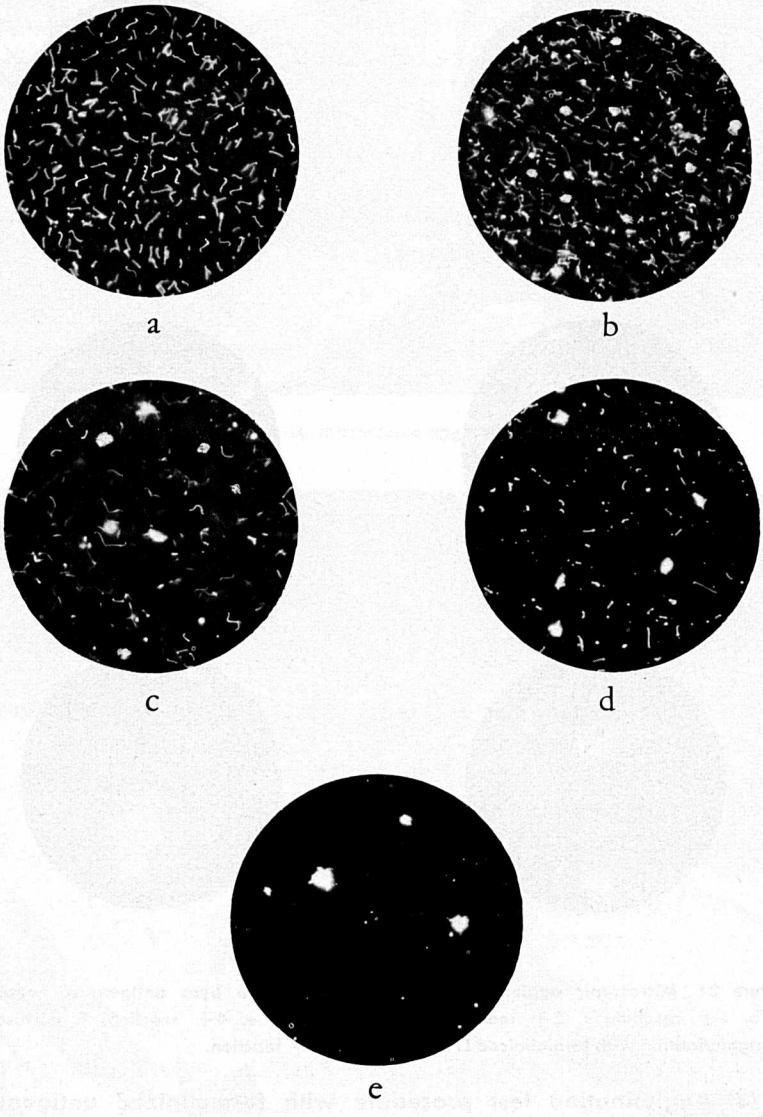



**Figure 22. Microscopic agglutination with live *Leptospira pyrogenes* antigen: a. negative; b. 1+ reaction; c. 2+ reaction; d. 3+ reaction; e. 4+ reaction.**

### C. Identification of Leptospiral Serotypes

The present system of identification and classification of leptospires is based upon antigenic analysis. Since knowledge of the bionomics of leptospires is not sufficient to classify different antigenic strains as species according to the codes of nomenclature, Wolff and Broom (*57*) proposed the term "serotype" to designate the basic taxon based on the agglutinogens of the leptospires. This system has been accepted by the World Health Organization Study Group on Leptospirosis and the Leptospira Subcommittee of the International Committee on Bacteriological Nomenclature (*58*) and is considered the best available method. Based on this method, 60 leptospiral serotypes or subserotypes have been recognized or provisionally classified (table 4). More than 37 additional strains have been described but are not officially accepted.

**(1) Screening and grouping of unknown cultures.**—A group of two or more leptospiral serotypes which by their serological reactions show close antigenic relationships are considered as a *serogroup.* A preliminary classification as to serogroup may be obtained by first screening the unknown culture with a battery of standardized rabbit antiserums against 12 serotypes. The microscopic agglutination test with a live actively growing culture in Stuart's medium should be used. The antiserums should be prepared from type strains of: *L. ballum, L. canicola, L. icterohaemorrhagiae, L. bataviae, L. grippotyphosa, L. pyrogenes, L. autumnalis, L. pomona, L. sejroe, L. australis, L. hyos,* and *L. mini georgia.* If the culture appears negative or shows only a low titer against these antiserums, a secondary battery of five antiserums should be tested, including *L. javanica, L. butembo, L. borincana, L. djasiman,* and *L. celledoni.* A routine diagnostic laboratory should not attempt to go further than this tentative classification into serogroups. The culture should then be sent to a leptospira reference laboratory for definitive identification by cross-agglutinin-absorption procedures. In the United States, such a definitive service may be obtained in the WHO/FAO Leptospirosis Reference Laboratory, Walter Reed Army Institute of Research, Washington, D.C., and in the Communicable Disease Center, National Leptospirosis Reference Laboratory, Veterinary Public Health Section, Atlanta 22, Ga.

In performing the microscopic agglutination test, some leptospirosis reference laboratories (*57*) employ a different dilution schema. In this schema, the serum is diluted to provide final concentrations of 1:10, 1:30, 1:100, 1:300, 1:1,000, 1:3,000, etc. For preliminary screening, however, it is not necessary to include the first two dilutions.

**(2) Preparation of antiserums.**—Normal rabbits weighing 8 to 9 pounds are inoculated intravenously with successive doses of 1.0 ml, 2.0 ml, 4.0 ml, and 6.0 ml. amounts of a 4- to 6-day-old culture of the desired leptospiral strain grown in Fletcher's medium (*1*). The four injections are given at 5- to 7-day intervals. Six to seven days after the last inoculation, a small amount of blood is obtained for testing. If the homologous titer

TABLE 4

SEROTYPES AND SUBSEROTYPES OF LEPTOSPIRES
(Modified from WHO Zoonoses Report 1959)

| SEROGROUP | SEROTYPE | SUBSEROTYPE | TYPE STRAIN | SYNONYMS | SPECIES | COUNTRY | YEAR |
|---|---|---|---|---|---|---|---|
| icterohaemorrhagiae | icterohaemorrhagiae (AB) | icterohaemorrhagiae | M 20 | bonarensis | man | Japan | 1914 |
| | icterohaemorrhagiae (A) | incompleta | RGA | | man | France | 1915 |
| | naam | | Naam | | man | Indonesia | 1936 |
| | mankarso | | Mankarso | | man | Indonesia | 1938 |
| | sarmin | | Sarmiń | | man | Indonesia | 1930 |
| | birkini | birkini | Birkin | | man | Malaya | 1955 |
| | birkini | smithii | Smith | | man | Malaya | 1953 |
| | ndambari | | Ndambari | | man | Belgian Congo | 1947 |
| javanica | javanica | | Veldrat Batavia | 46 | rat | Indonesia | 1938 |
| | poi | | Poi | sorex | man | Italy | 1941 |
| | coxus | | Cox | | man | Malaya | 1954 |
| canicola | canicola | | Hond Utrecht IV | Kashirskii | canine | Netherland | 1933 |
| | schueffneri | | Vleermuis 90 C | | bat | Indonesia | 1938 |
| | benjamin | | Benjamin | | man | Indonesia | 1937 |
| | jonsis | | Jones | | man | Malaya | 1953 |
| | sumneri | | Sumner | | man | Malaya | 1955 |
| | malaya | | H-6 | | man | Malaya | 1951 |
| ballum | ballum | ballumensis | Mus 127 | | mouse | Denmark | 1943 |
| | ballum (AB) | castellonis | Castellon 3 | | man | Spain | 1955 |
| pyrogenes | pyrogenes | | Salinem | febrilis okinawa | man | Indonesia | 1924 |
| | zanoni (australis B) | | Zanoni | | man | Australia | 1935 |
| | abramis | | Abraham | | man | Malaya | 1954 |
| | biggis | | Biggs | | man | Malaya | 1954 |
| | hamptoni | | Hampton | | man | Malaya | 1954 |
| cynopteri | cynopteri | | 3522 C | | bat | Indonesia | 1938 |
| | butembo | | Butembo | | man | Belgian Congo | 1946 |
| sentot | sentot | | Sentot | | man | Indonesia | 1937 |
| autumnalis | autumnalis (AB) | autumnalis | Akiyami A | | man | Japan | 1925 |
| | autumnalis (A) | rachmati | Rachmat | | man | Indonesia | 1923 |
| | bangkinang | | Bangkinang I | | man | Indonesia | 1929 |
| | mooris | | Moores | | man | Malaya | 1954 |
| djasiman | djasiman | | Djasiman | | man | Indonesia | 1937 |
| australis | australis (australis A) | | Ballico | akiyami C hebdomadis C | man | Australia | 1937 |
| | muenchen | | Munchen C-90 | | man | Germany | 1942 |
| | esposito | | Esposito | | man | Australia | 1954 |

TABLE 4 (Continued)

| SEROGROUP | SEROTYPE | SUBSEROTYPE | TYPE STRAIN | SYNONYMS | SPECIES | COUNTRY | YEAR |
|---|---|---|---|---|---|---|---|
| pomona | pomona | | Pomona | australis C<br>type II<br>suis<br>monjakov<br>DVB | man | Australia | 1937 |
| grippotyphosa | grippotyphosa | | Moskva V | andaman B<br>type CH-3<br>bovis<br>geffeni<br>vituloeum | man | Russia | 1928 |
| hebdomadis | hebdomadis | | Hebdomadis | akiyami B<br>hebdomadis B | man | Japan | 1918 |
| | medanensis | | Hond HC | | dog | Indonesia | 1929 |
| | wolfii | | 3705 | | man | Indonesia | 1937 |
| | hardjo | | Hardjoprajitno | | man | Indonesia | 1938 |
| | mini | mini | Sari | Barthelemy | man | Italy | 1941 |
| | mini | szwajizak | Szwajizak | | man | Australia | 1932 |
| | kremastos | | Kremastos | | man | Australia | 1952 |
| | kabura | | Kabura | | man | Belgian Congo | 1946 |
| | jules | | Jules | Tsango<br>Kambori, messo | man | Belgian Congo | 1955 |
| | haemolytica | haemolytica | Marsh | | man | Malaya | 1954 |
| | haemolytica | ricardi | Richardson | | man | Malaya | 1954 |
| | worsfoldi | | Worsfold | | man | Malaya | 1953 |
| | sejroe | | M 84 | | man | Denmark | 1937 |
| | saxkoebing | | Mus 24 | nin | mouse | Denmark | 1942 |
| | borincana | | HS-622 | | man | Puerto Rico | 1951 |
| bataviae | bataviae | | Swart | mitis<br>mino<br>oryzeti | man | Indonesia | 1932 |
| | paidjan | | Paidjan | | man | Indonesia | 1939 |
| semaranga | semaranga | | Veldrat S 173 | | rat | Indonesia | 1937 |
| andamana | andamana | | CH 11 | | man | Andaman Islands | 1931 |
| hyos | hyos (mitis johnson) | hyos | Mitis Johnson | perepelitsin<br>tarassovi<br>DVA T 384 | man | Australia | 1940 |
| | hyos | bakeri | LT 79 | | opossum | United States | 1955 |
| celledoni | celledoni | celledoni | Celledoni | | man | Australia | 1952 |
| | celledoni (II) | whitcombi | Whitcomb | | man | Malaya | 1954 |

of the serum is at least 1: 12,800, the rabbits are exsanguinated. The serum is harvested, an equal volume of sterile glycerine added as a preservative, and the serum stored at 4° C. If the titer is low, another injection of 4 to 6 ml. of antigen may be given. However, it is frequently necessary to try a new rabbit, as some are poor antibody producers. If any portion of the serum is to be used for fluorescent antibody studies, it should be filtered through a bacteriological filter, dispensed in small amounts, and frozen or lyophilized.

Many laboratories use the above procedure with a culture in liquid Stuart's medium of the desired serotype, killed by the addition of 0.3 percent formalin. Serums prepared by this method usually show lower titers and more cross-agglutination. However, antiserums prepared with killed antigens have proved satisfactory and, if adequate animal isolation facilities are not available, this method may be used.

**(3) Agglutinin-absorption technique.**—The cross agglutination screening test will indicate the serogroup to which the strain belongs. It must then be tested with antiserums against all members of that serogroup. Agglutinin-absorption tests should be performed with each serotype in which cross reactions equal to 3 percent of the homologous titer have been obtained.

Antigens for absorption studies are prepared from 4- to 6-day-old cultures grown in Stuart's medium in 500-ml. amounts. Thirty-two-ounce screwcapped prescription bottles are convenient for this purpose. For best results, these bottles are slanted at a 45° angle during incubation. The cultures are killed by the addition of formalin to provide a final concentration of 0.5 percent. The antigen is then centrifuged at 5,000 times gravity in a high-speed angle-head centrifuge for 25 minutes. The supernatant is discarded and the leptospires from 250-ml. portions of culture resuspended in 1 to 2 percent of the original culture volume with a 0.25 percent formalin buffered saline solution. One part of a 1: 10 dilution of antiserum is added to four parts of the absorbing antigen,[1] mixed thoroughly, incubated in a 50° C. water bath for 2 hours and overnight at 28°–30° C. The cells are removed by centrifugation and the absorbed serum tested for the presence of homologous antibody. It may be necessary, however, to perform a second absorption. To avoid further dilution, the serum is added to packed cells from 250 ml. of killed antigen. The serum-cell mixture is incubated 2 to 3 hours at 37° C. and again tested for the presence of homologous antibody. Absorptions are considered complete when agglutinins are completely removed by the homologous antigen. Microscopic agglutination tests with absorbed serums are performed with killed antigens. Final twofold serum dilutions of 1: 100 through 1: 25,600 are used. Incubation and reading of the tests is described in B(3).

[1] Wolff and Broom (*57*) recommend for absorption the addition of one part of serum that has been diluted to a standard titer of 1: 3,000 to nine parts packed cells.

**(4) Interpretation of agglutinin-absorption findings.**—"Two strains are considered to belong to different serotypes if, after cross-absorption with adequate amounts of heterologous antigen, 10 percent or more of the homologous titer regularly remains in each of the 2 antisera" (*57*). If, after repeated tests, however, less than 10 percent of the homologous titer remains in one antiserum and 10 percent or more in the other antiserum after cross-absorption with adequate amounts of heterologous antigen, a strain is considered as a *subserotype* within a serotype.

The Joint WHO/FAO Expert Committee on Zoonoses (*58*) recommends that, before a new serotype is accepted as valid, the work should either be verified in one of the WHO/FAO leptospirosis reference laboratories, or, if the work has been done in a reference laboratory, the protocols of agglutination and agglutinin-absorption should be reviewed in a second reference laboratory.

**WHO/FAO LEPTOSPIROSIS REFERENCE LABORATORIES***

1. Laboratory of the Queensland Department of Health and Home Affairs, Brisbane, Queensland, Australia.
2. Instituto Superiore di Sanita, Rome, Italy.
3. Department of Viral and Rickettsial Diseases, National Institute of Health, Tokyo, Japan.
4. Institute for Tropical Hygiene and Geographical Pathology (Royal Tropical Institute), Amsterdam, Netherlands.
5. The Wellcome Laboratories of Tropical Medicine, London, England.
6. Division of Veterinary Medicine, Walter Reed Army Institute of Research Washington 12, D.C., USA.

*Joint WHO/FAO Expert Committee on Zoonoses, Second Report, WHO Techn. Rep. Ser., No. 169, p. 72, 1959.

## APPENDIX

### 1. Fletcher's Medium (8)

**Formula**

| | |
|---|---|
| Peptone | 0.3 gm. |
| Beef extract | 0.2 gm. |
| Sodium chloride | 0.5 gm. |
| Agar | 1.5 gm. |
| Distilled water | 920 mls.[1] |
| Sterile rabbit serum | 8–10 percent. |

[1] If the distilled water is acid, it should be buffered before use to pH 7.4 with Sorensen's buffer.

Sterilize by autoclaving the base for 15 minutes at 15 pounds pressure. Cool to 50° C. and add 8 to 10 percent sterile rabbit serum that has been warmed to 50° C. in a water bath. Mix to achieve a uniform solution. Dispense aseptically, and inactivate the whole medium at 56° C. for 1 hour on the day it is dispensed and 1 hour on the following day. The pH of this medium need not be readjusted and should be in the range 7.6 to 8. This medium is available in dehydrated form from Difco and BBL Laboratories.

The completed medium should be incubated for 24 hours at 37° C. and for 24 hours at 30° C. to check for sterility. To check the medium for ability to support growth of leptospires, inoculate minimal amount (0.1 to 0.2 ml.) of *actively growing* stock strains into each lot of medium. At least 5, and preferably 12, serotypes should be used.

### 2. Stuart's Medium (7)

**Formula**

| | |
|---|---|
| Asparagine | 0.132 gm. |
| Ammonium chloride | 0.268 gm. |
| Magnesium chloride $.6H_2O$ | 0.406 gm. |
| Sodium chloride | 1.808 gm. |
| Disodium phosphate | 0.666 gm. |
| Monopotassium phosphate | 0.087 gm. |
| Phenol red | 0.01 gm. |
| Distilled water | 995 ml. |
| Sterile rabbit serum | 8–10 percent. |
| Glycerine | 5.0 ml. |

Sterilize by autoclaving at 15 pounds pressure for 15 minutes. Cool the base to less than 50° C. and add 8 to 10 percent sterile rabbit serum. Mix to obtain uniform solution and dispense aseptically. The pH of this medium should be from 7.4 to 7.6. This medium is available in a dehydrated form from Difco.

The completed medium should be incubated for 24 hours at 37° C. and for 24 hours at 30° C. to check for sterility. To check the medium for ability to support growth of leptospires, inoculate minimal amount (0.1 to 0.2 ml.) of *actively growing* stock strains into each lot of medium. At least 5, and preferably 12, serotypes should be used.

For plates, 1 percent agar may be added to this medium (*51*).

### 3. Cox's Medium (50)

Dissolve 2 gm. Tryptose-phosphate (Tryptose or trypticase may be substituted) and 10 gm. Difco agar in 900 ml. distilled $H_2O$ in a steamer or over a burner, making certain there is no appreciable reduction in volume during heating. The pH of this base does not have to be adjusted. Dispense in 90-ml. volumes in pyrex, screwcap, milk dilution bottles and autoclave. This base may be stored at room temperature. When needed, the bottles of agar base may be melted, cooled to 50° C., and after adding aseptically 10 ml. of sterile rabbit serum to each bottle, 3 Petri plates may be poured. The bottles of medium may be pasteurized at 56° C. in a water bath for 30 to 60 minutes before pouring, if desired. At least 30 ml. of the completed medium should be poured into each 100 mm. Petri dish. The rabbit serum, slightly hemolyzed, should be sterilized by filtration through a Seitz filter (ST–3) which has been previously flushed with buffered saline. Incubate plates overnight at 37° C. to check for sterility.

### 4. Rabbit Serum

Occasionally, the serum from a single rabbit may fail to support growth of leptospires. For this reason, *pooled rabbit serum* should be used for all leptospiral media. It is desirable to pool serum from at least 20 rabbits, and preferably more. In some areas, rabbit blood may be obtained from commercial slaughter plants where large numbers of animals are killed. The serum should be sterilized by filtration through a Seitz filter with sterilizing pad (ST–3). About 50 ml. of buffered saline should be put through the filter before the serum to neutralize the filter pad. Prior to use, each lot of pooled rabbit serum should be checked for the presence of leptospiral antibodies by the macroscopic or microscopic agglutination tests.

### 5. Buffered Saline Solution

Sterile buffered saline solution;

| | *Ml.* |
|---|---|
| Physiological saline solution (0.85 per cent) | 1,840 |
| Sorensen's buffer [1] | 160 |
| | 2,000 |

[1] Sorensen's buffer:

| | |
|---|---|
| Sodium phosphate (anhydrous) ($Na_2PHO_4$) | 8. 33 gm. |
| Potassium phosphate (Monobasic) ($KH_2PO_4$) | 1. 09 gm. |
| Distilled water | 1 liter. |

Final pH should be 7.6.

Dispense in 16 oz. screw capped bottles, 200 ml. per bottle.

Autoclave at 15 lbs. pressure for 15 minutes. Determine the final pH after autoclaving. It should be pH 7.5.

**6. Congo Red Negative Stain (25):**

a) Congo Red, 2% aqueous solution

b) Ethanol, 95% containing 1% (v/v) concentrated hydrochloric acid.

Prepare smear by mixing a drop of culture of leptospires on a slide with a drop of 2 percent aqueous Congo Red. Let smear dry, then cover slide with 95 percent ethanol containing 1 percent concentrated hydrochloric acid and leave to dry. To examine, use oil immersion lens and bright field lighting. The leptospires appear unstained against a dark blue background.

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# ERRATA SHEET

## LEPTOSPIROSIS

Epidemiology, Clinical Manifestations
in Man and Animals,
and
Methods in Laboratory Diagnosis
PHS Publication No. 951, June 1962
by
Mildred M. Galton, Sc.M.
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The authors feel that the attached photographs give greater clarity and detail needed by anyone trying to duplicate the techniques shown. It is recommended that the figures be inserted by recipients of PHS Publication No. 951, June 1962, as follows:

On page 38 – Figure 7. Inoculation of blood into Fletcher's medium.

On page 41 – Figure 9. Obtaining urine by bladder tap.

On page 43 – Figure 10. Removal of kidney from a guinea pig at necropsy.

On page 51 – Figure 16. Rotation of serum-antigen mixture for slide agglutination test.
and
Figure 17. Reading macroscopic slide agglutination test.

On page 56 – Figure 19. Setting up microscopic agglutination test.
and
Figure 20. Reading microscopic agglutination test.

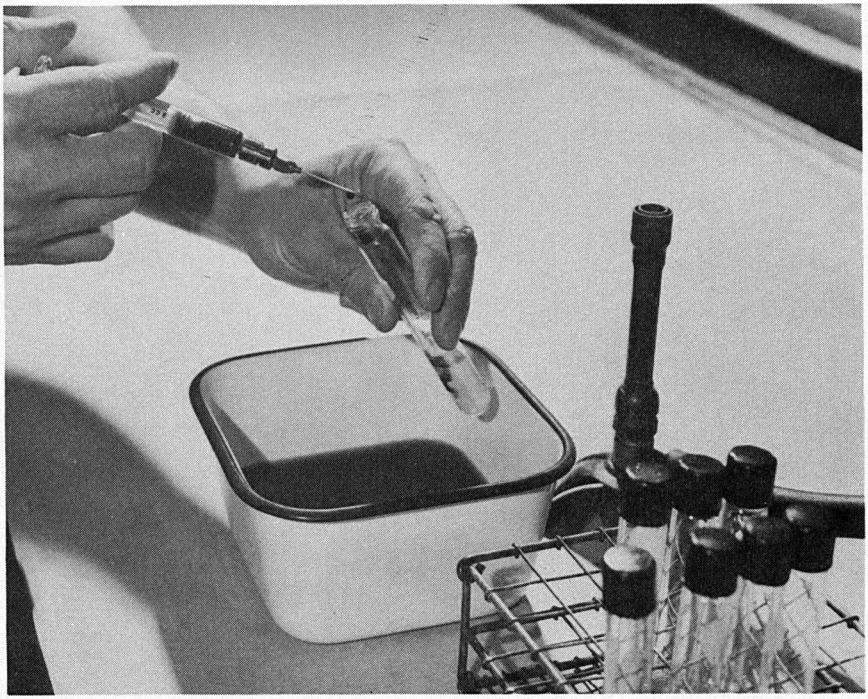

**Figure 7. Inoculation of blood into Fletcher's medium.**

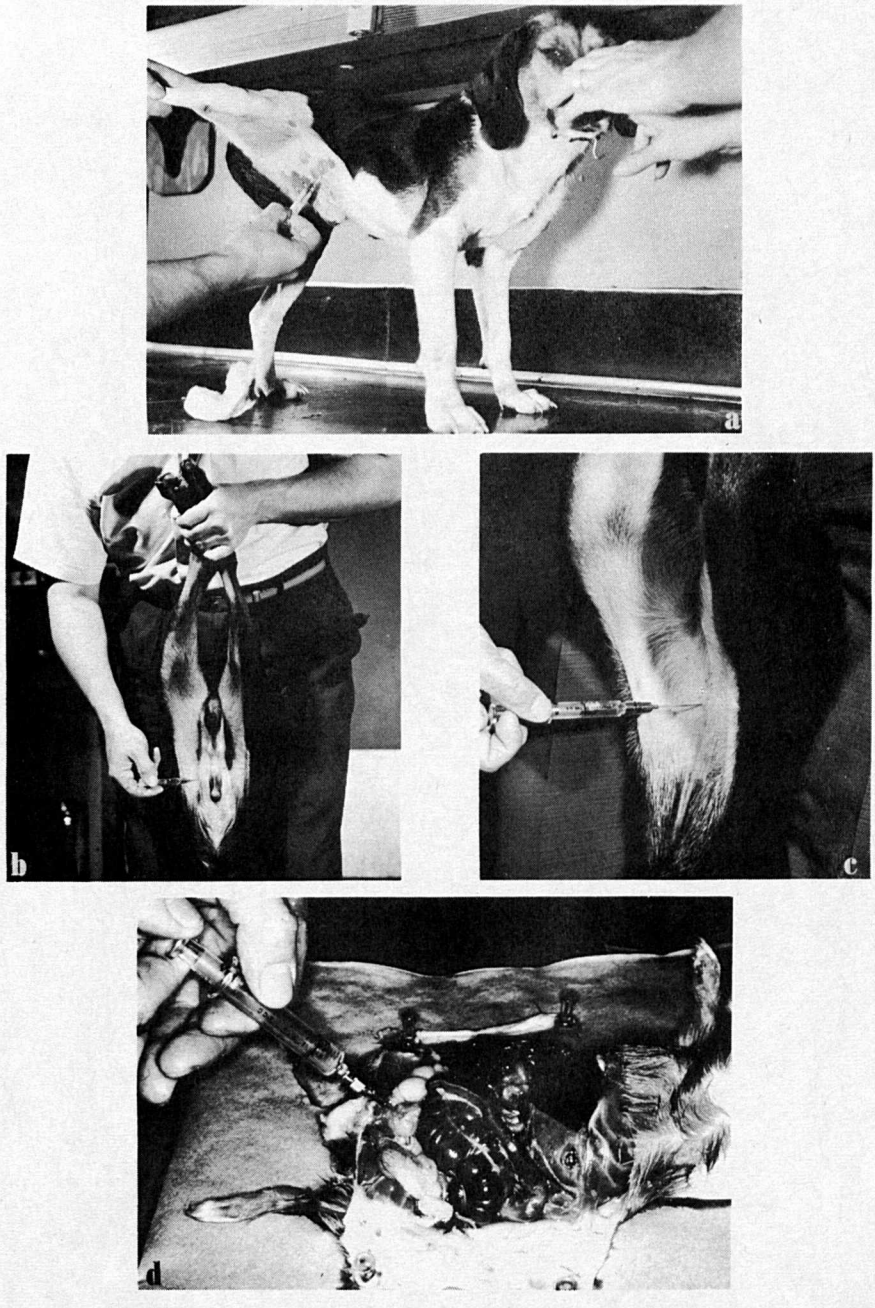


Figure 9. Obtaining urine by bladder tap. a. From a male dog in standing position; b. From male dog in a suspended position; c. From a female dog in a suspended position; d. From a guinea pig at necropsy.

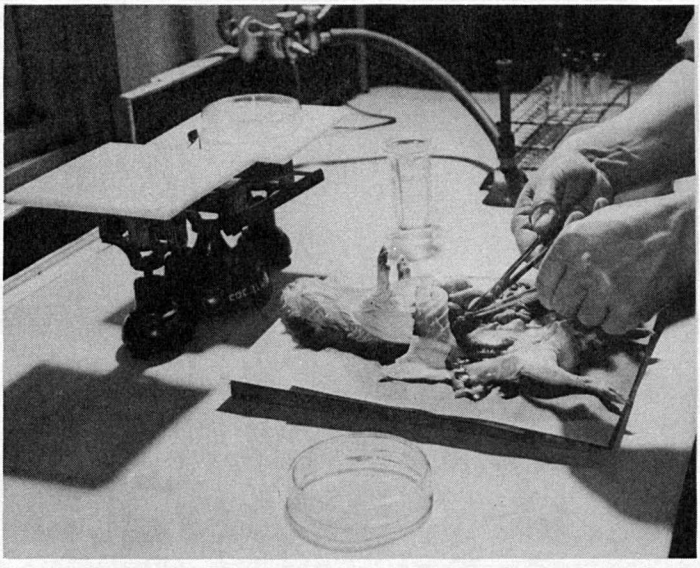

Figure 10. Removal of kidney from a guinea pig at necropsy.

Figure 16. Rotation of serum-antigen mixture for slide agglutination test.

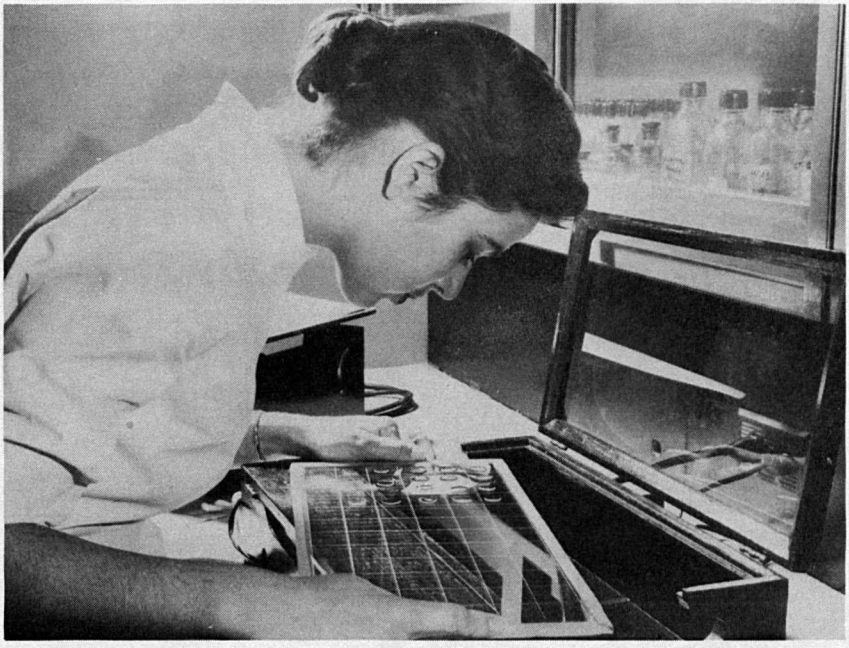

Figure 17. Reading macroscopic slide agglutination test.

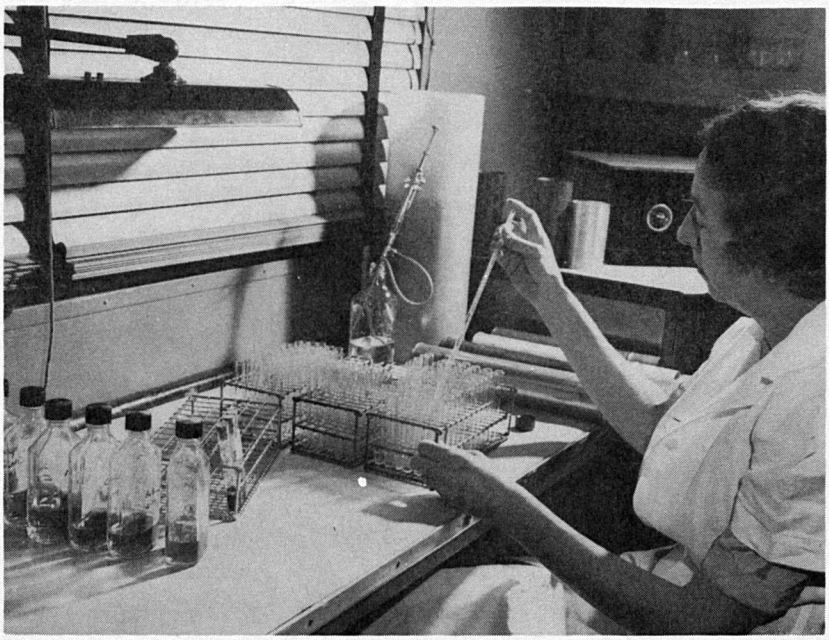

Figure 19. Setting up microscopic agglutination test.

Figure 20. Reading microscopic agglutination test.